

Vitamin C Status, Oxidative Stress, Hyperglycaemia and Endothelial Function in Critically Ill Patients with Septic Shock: an Observational Study

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Vitamin C status, oxidative stress, hyperglycaemia and endothelial function in critically ill patients with septic shock: an observational study

ABSTRACT

Septic shock is associated with oxidative stress, reduced levels of plasma vitamin C and stress hyperglycaemia – all factors that may influence endothelial, and therefore, organ function. Vitamin C is an important antioxidant in human plasma; and it has been implicated in maintaining normal endothelial function during oxidative stress. The vitamin C status of critically ill patients in South African ICUs has not been well investigated; neither has the relationship between vitamin C status, oxidative stress, hyperglycaemia and endothelial function been studied in this patient group.

In a prospective, cross-sectional study investigating these factors in critically ill patients with septic shock on inotropic support, serial blood samples from 25 patients were taken at days zero and one, following inotrope initiation, and on day seven after inotrope cessation. These samples were analysed for plasma vitamin C, thiobarbituric acid-reactive substances (TBARS) – as a biomarker of oxidative stress – and soluble vascular cell adhesion molecule-1 (sVCAM-1), and E-selectin, as markers of endothelial dysfunction. The plasma glucose to vitamin C ratios were also calculated. Daily clinical measures in the patients included Sequential Organ Failure Assessment (SOFA) score, mean arterial blood pressure, blood glucose, fluid balance and inotropic support. The clinical outcomes were recorded.

Septic shock patients had low levels of plasma vitamin C at baseline, which persisted up to day seven – after the resolution of septic shock. There was also evidence of oxidative stress, marked by increased levels of plasma TBARS. At the baseline, markers of oxidative stress were statistically significantly higher in non-survivors than in the survivors of septic shock. Plasma glucose to vitamin C ratios were raised on all the study days. In non-survivors, the maximum blood glucose correlated negatively with vitamin C levels.

The bio-markers of endothelial dysfunction (sVCAM-1 and E-selectin) were raised at the baseline. Soluble VCAM-1 levels fell significantly on day one, and were normalized on day seven; while the E-selectin levels were raised on day one; and they further increased significantly up to day seven. Oxidative stress and endothelial dysfunction were associated with increased organ dysfunction, measured by the SOFA score. Increased oxidative stress was associated with both increased duration of inotropic support, and the increased requirements for intravenous fluids.

This study has, therefore, demonstrated that low vitamin C status and oxidative stress persisted up to seven days after the resolution of septic shock. Increased oxidative stress was associated with increased endothelial damage; and both of these factors correlated positively with the severity of organ dysfunction. We also demonstrated

that increased oxidative stress was associated with increased intravenous fluid, and with prolonged inotrope requirements.

The study confirms low vitamin C status and the association of oxidative stress with endothelial dysfunction and multiple organ failure in septic shock. It discusses the need for further studies to explore the impact of high dosage vitamin C supplementation on the outcome in this patient group.

ABBREVIATIONS

μM	Micromolar
Ab	Absorbance of the background well with ascorbate oxidase
ANOVA	Analysis of variance
APACHE	Acute Physiology and Chronic Health Evaluation
At	Absorbance of the total antioxidant well
BH_4	Tetrahydrobiopterin
BHT	Butylated hydroxytoluene
BMI	Body Mass Index
CA	California
CLP	Caecal ligation and perforation
CO	Cardiac output
DHA	Dehydroascorbic
DNA	Deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ET	Endothelin
FHS	Faculty of Health Sciences
FiO_2	Fraction of Inspired Oxygen
FIP	Faeces into the peritoneum
FRASC	Ferric Reducing Ascorbate
GA	Georgia
GCP	Good Clinical Practice
GLUT1	Glucose transporter1
GLUT3	Glucose transporter 3
GPx	Glutathione peroxidase
H_0	Null hypothesis
H_A	Alternative hypothesis
HPLC	High-performance liquid chromatography
HREC	Human Research Ethics Committee
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cells

ICAM-1	Intercellular adhesion molecule-1
ICH	International Conference for Harmonisation
ICU	Intensive care unit
IEC	Independent Ethics Committee
IL-1 β	Interleukin 1-beta
Il-6	Interleukin -6
iNOS	Inducible nitric oxide synthase
IQR	Interquartile range
IRB	Institutional Review Board
IU	International units
IV	Intravenous
Kg	Kilogram
LPS	Lipopolysaccharide
m ²	Square metre
MAP	Mean arterial pressure
mcg	Micrograms
ml	Millilitre
mmHg	Millimetres of Mercury
mmol/l	Millimoles per litre
MO	Missouri
MSOF	Multisystem organ failure
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng/ml	Nanograms per millilitre
NICE-SUGAR	Normoglycaemia in Intensive Care Evaluation–Survival Using Glucose Algorithm Regulation
nmol/ml	Nanomoles per millilitre
NO	Nitric oxide
°C	Degrees Celsius
PaCO ₂	Partial pressure of arterial carbon dioxide
PAF	Platelet activating factor
PAO ₂	Partial pressure of oxygen in arterial blood
PAMPs	Pathogen-associated molecular patterns
PDGF	Platelet-derived growth factor

PRRs	Pattern recognition receptors
RDA	Recommended daily allowance
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SD	Standard deviation
SIRS	Systemic inflammatory response syndrome
SOD	Superoxide dismutase
SOFA	Sequential Organ Failure Assessment
sVCAM-1	Soluble cell adhesion molecule-1
SVCT2	Sodium dependent vitamin C transporter 2
SVR	Systemic vascular resistance
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TLRs	Toll-like receptors
TMB	Tetramethylbenzidine
TNF- α	Tumour necrosis factor-alpha
TPN	Total Parenteral Nutrition
UCT	University of Cape Town
USA	United States of America
V	Sample volume added into the reaction well

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CHAPTER 1: LITERATURE REVIEW

1.1. Critical illness

Critical-care medicine is the management of severe illness associated with organ dysfunction, such as acute respiratory failure, in patients who are at risk of imminent death (Adhikari et al., 2010; Ehlenbach et al., 2010). In the early days of intensive-care medicine, the emphasis was mainly on the mechanical ventilation of patients, such as those suffering from poliomyelitis (IBSEN, 1954).

The discipline has now advanced in the understanding of the pathophysiology of dysfunctional organs and the development of the respective supportive technology (Adhikari et al., 2010). Septic shock is one of the common disease processes in critical illness; and it is a major cause of morbidity and mortality worldwide (Roman-Marchant et al., 2004).

1.2. Septic shock

Table 1 describes the definitions of systemic inflammatory response syndrome (SIRS), sepsis, severe sepsis and septic shock. Sepsis is defined as SIRS that is characterized by abnormalities in two or more clinical features, including body temperature, heart rate, respiratory function and peripheral leukocyte count, in the presence of, or as a result of, suspected or proven infection (Bone et al., 1992).

When severe, sepsis can be complicated by the presence of cardiovascular organ dysfunction, acute respiratory distress syndrome or other organ dysfunction. Septic shock occurs when severe sepsis is complicated by acute circulatory failure, which is characterized by persistent arterial hypotension – despite adequate fluid resuscitation, or by tissue hypo-perfusion manifested by a raised lactate concentration that is unexplained by other causes (Dellinger et al., 2013).

It is important to note that 40% of severe septic cases progress to septic shock (López-Bojórquez, Dehesa & Reyes-Terán, 2004). Such patients require ICU management including organ support; and the condition has increased morbidity and mortality (Angus & Tom, 2013; Kumar et al., 2011). Those who survive often have

impaired physical or neurocognitive functioning, mood disorders, a low quality of life; and they have the additional risk of mortality (Angus et al., 2001).

Table 1: Definition of SIRS/Sepsis

SIRS (Systemic Inflammatory Response Syndrome)	Two or more of the following criteria:
	- Temperature < 36 °C or > 38 °C
	- Heart rate > 90 beats per minute
	- Respiratory rate > 20 breaths per minute or PaCO ₂ < 32 mmHg
	- White blood cell count > 12000 x10 ⁹ /L < 4000 x10 ⁹ /L, or > 10% immature (band) forms
Sepsis	Documented infection together with two or more SIRS criteria above
Severe Sepsis	Sepsis associated with organ dysfunction
Septic Shock	Sepsis with refractory hypotension or hypoperfusion abnormalities in spite of adequate fluid resuscitation

Derived from Bone RC, Balk RA, Cerra FB, et al. (June 1992)

Currently, guidelines on the management of severe sepsis in ICU include: Initial resuscitation with fluids or blood products, appropriate antibiotic therapy (preferably within the first hour of recognition of septic shock), diagnosis of the cause of the sepsis and controlling the source of sepsis, further infection prevention and hospital-based performance-improvement efforts in the management of severe sepsis (Dellinger et al., 2013).

Other supportive measures include vasopressor and inotropic-vasopressor therapy, glycaemic control, prophylaxis for deep-vein thrombosis, prophylaxis for stress ulceration in patients with risk of bleeding, oxygen supplementation and mechanical ventilation, as well as other organ-support treatments, as may be indicated (Dellinger et al., 2013). Despite the advances in its management, septic shock is still associated with high morbidity and mortality. Statistics show that 20% – 30% of patients with septic shock still die – even in settings where aggressive antibiotic therapy and advanced life support are provided (Kethireddy & Kumar, 2012; Labelle et al., 2012). This could possibly be due to the complex pathophysiology associated with it; and this calls for the exploration of additional management approaches.

1.2.1. Pathophysiology and consequences of septic shock

The pathogenesis of septic shock that leads to multi-organ dysfunction is multifactorial and complex (López-Bojórquez, Dehesa & Reyes-Terán, 2004). Figure 1 below describes the steps that occur in uncontrolled sepsis – leading to septic shock.

The cascade of events begins with a focus of infection (step 1, Figure 1), which could be in any organ where there is a release of protein fragments or immunogens from viruses, bacteria or fungi into the blood stream (Crowley, 1996). The causative organism of the infection may then enter the blood stream, where the innate immune system recognises evolutionarily conserved structures on pathogens, termed pathogen-associated molecular patterns (PAMPs) (Mogensen, T.H., 2009) (step 2 Figure 1). PAMPs are recognised through a limited number of germ line-encoded pattern recognition receptors (PRRs) including the family of Toll-like receptors (TLRs) (Mogensen, T.H., 2009). Several studies, mainly focusing on gram-negative bacterial sepsis have shown that lipopolysaccharide is shed from the cell wall of pathogens, such as *E. coli* (Bayston & Cohen, 1990; Glauser et al., 1991; Rietschel & Brade, 1992). However, lipoteichoic acid from gram-positive bacteria and ergosterols from fungi have also been described (López-Bojórquez, Dehesa & Reyes-Terán, 2004).

The lipid A portion of the lipopolysaccharide molecule is the part involved in the binding of the molecule to virtually all cells once they are released into the blood. (Bayston & Cohen, 1990; Crowley, 1996; Glauser et al., 1991; Rietschel & Brade, 1992). As depicted in step 3 of Figure 1, the binding of the polysaccharides to the endothelium, monocytes or macrophages induces the production of chemical mediators in the pathogenesis of septic shock. When activated, the monocyte/macrophage system secretes a variety of soluble factors, such as peptide hormones with pro-inflammatory activity and inflammatory cytokines [tumour-necrosis factor-alpha (TNF- α), interleukin 1-beta (IL-1 β) and interleukin- 6 (IL-6)] that in turn activate other cells (López-Bojórquez, Dehesa & Reyes-Terán, 2004)

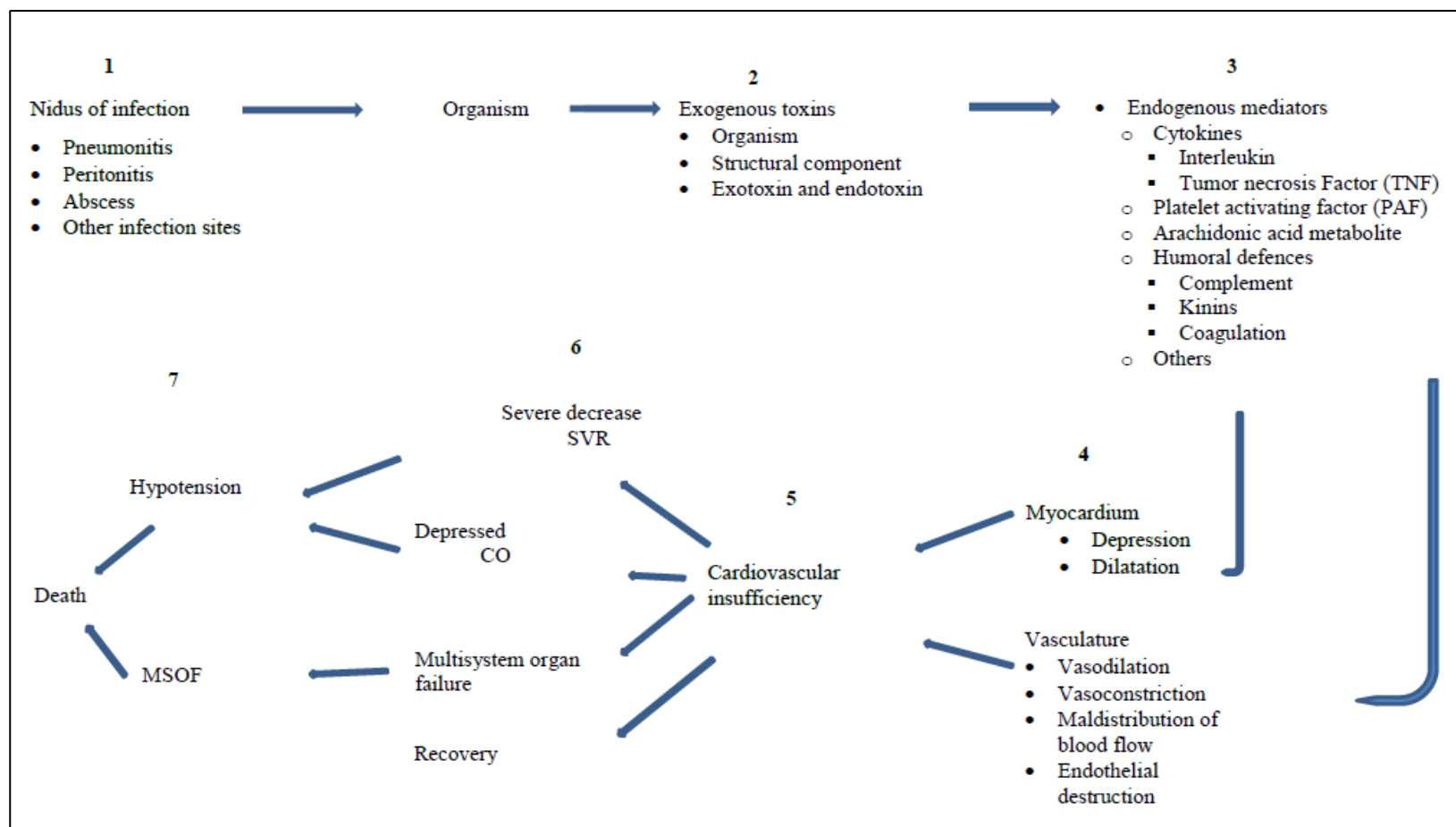


Figure 1. Sequence of pathogenetic steps leading from nidus of infection to cardiovascular dysfunction and shock during human sepsis

SVR = Systemic vascular resistance; CO = cardiac output; MSOF = multisystem organ failure

It is these cytokines that directly or indirectly cause serial changes at cellular levels in the vascular and immune system to begin with – and then to the rest of the tissues (Fantuzzi et al., 2000). Production of the endogenous mediators following sepsis and sepsis itself affects the heart and the systemic circulation.

In the heart, sepsis-induced myocardial dysfunction is common, and corresponds to the severity of sepsis, as shown in step 4 of Figure 1 (Furian et al., 2012). The mechanisms of this process include the attenuation of the adrenergic response at the cardiomyocyte level, the alterations of intracellular calcium trafficking, and the blunted calcium sensitivity of contractile proteins (Rudiger & Singer, 2013). These changes are mediated by cytokines (Rudiger & Singer, 2013). *In vitro* studies have supported this by showing that serum from septic patients decreases myocyte contractile function; and these studies have also indicated the presence of myocardial-depressant mediators, including nitric oxide (NO) (Kumar et al., 2001; Lupia et al., 2010; Merx & Weber, 2006).

On the systemic circulation, septic shock is associated with the maldistribution of intravascular blood volume (Crowley, 1996; Nduka & Parrillo, 2011). This could be explained in part as the result of generalised vasodilation that results from the hyporesponsiveness of blood vessels to the sympathetic system – leading to the loss of vascular tone (Biesalski & McGregor, 2007; McGregor & Biesalski, 2006). The reduced responsiveness to epinephrine and norepinephrine could be explained by three possible mechanisms. Firstly, there is the altered affinity of receptors to these hormones (Boyd, Stanford & Chernow, 1989). Secondly, there is a reduction in the number of adrenergic receptors – as a response to the activity of TNF- α (Silverman et al., 1993). Thirdly, there is evidence suggesting that there are intracellular defects that render the response to the hormones dysfunctional (Boyd, Stanford & Chernow, 1989; Silverman et al., 1993).

The vasodilation is further enhanced by the production of abnormally high levels of NO by the endothelial cells, through the inducible Nitric Oxide synthase system (iNOS). The iNOS is mediated by nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), and is induced under septic conditions (Tyml, Li & Wilson, 2008a; Wilson & Wu, 2012; Wu, Wilson & Tyml, 2003). The ultimate

results of the vasodilation are an increased cardiac output, decreased perfusion in some organs, lowered effective blood volume, tachycardia and hypotension (Crowley, 1996). These are the clinical features observed in septic shock. Studies using animal models of sepsis have confirmed that the effects of excessive NO production contributes – not only to the generalised hypotension – but also to the myocardial dysfunction that occurs in sepsis (Rudiger & Singer, 2007).

One of the important effects of cardiovascular insufficiency (steps 5 and 6 of Figure 1) is multisystem-organ failure, following the generalised hypotension and reduced tissue perfusion. This leads to the increased morbidity; and although some patients survive, up to 50% do not survive septic shock (Labelle et al., 2012).

Apart from the mechanisms in the development of septic shock, as described above, the production of free radicals, which contribute to oxidative stress, is also an important pathophysiological phenomenon in severe sepsis.

1.3. Free radical formation, antioxidants and oxidative stress

1.3.1. Free radical formation and antioxidants

Free radicals are defined as molecules possessing one or more unpaired electrons; but they can also be seen as a fragment of a molecule (Halliwell, 1994). Important free radicals in pathological processes include oxygen free radicals, and reactive oxygen species (ROS) i.e. superoxide anion, hydrogen peroxide and the hydroxyl radical, as well as the reactive nitrogen species (RNS) e.g. nitric oxide and peroxynitrite, a product of the reaction between superoxide and nitric oxide (Halliwell, 1997; Halliwell, 2012).

Under normal homeostasis, free radicals may be generated in regulated amounts to take part in some processes, such as in proliferative signalling; and they may also be produced by phagocytes – as a measure of the host's defense against infection (Babior, 1978). In other cases, free radicals may also be incidental products, as in the case of the mitochondrial electron-transport chain, where some electrons may escape from electron carriers directly to the oxygen forming superoxide radical (Berger, 2005; Halliwell, 1997). When uncontrolled, free radicals – by oxidation – may

damage biologically relevant molecules, such as DNA, RNA, proteins, carbohydrates and the unsaturated fatty acids of the cell membranes (Berger, 2005; Goodyear-Bruch & Pierce, 2002) thereby triggering cell injury and tissue dysfunction (Lovat & Preiser, 2003). The potential harmful effects of these free radicals are opposed by antioxidant mechanisms.

Antioxidants are substances, which inhibit or delay the oxidation, while present in small amounts (Halliwell & Gutteridge, 1990). Antioxidants scavenge free radical intermediates and inhibit other oxidation reactions by being oxidized themselves, so antioxidants are often reducing agents (Sies & Stahl, 1995). Antioxidants exist in two categories, namely non-enzymatic and enzymatic systems. Non-enzymatic antioxidants include micro-nutrients, such as vitamin C, vitamin E and beta-carotene, but also endogenous molecules, such as glutathione, bilirubin and albumin (Berger, 2005). Enzymatic antioxidant systems include superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx), respectively, where trace elements, such as copper, manganese, zinc, iron and selenium are required for their activity (Lovat & Preiser, 2003). When oxidants are produced in excess, antioxidants cannot quench all the free radicals that are produced; and consequently, oxidative stress occurs.

1.3.1.1. The micronutrient antioxidant vitamin C

Vitamin C is an important antioxidant micronutrient. It is a major water-soluble antioxidant that works as the first line of defence against free radical action in plasma and whole blood. It is an essential nutrient in humans and other animals, due to the absence of the enzyme l-gulonolactone oxidase required for its synthesis from glucose (Linster & Schaftingen, 2007). The term vitamin C refers to both ascorbic acid and dehydroascorbic acid (DHA) (Seno et al., 2004).

The biological functions of ascorbic acid as an antioxidant are based on its ability to provide reducing equivalents for a variety of biochemical reactions. The vitamin readily scavenges ROS and RNS (e.g., hydroxyl, peroxy, superoxide, peroxyxynitrite, and nitroxide radicals) as well as singlet oxygen and hypochlorite (Buettner, 1993; Halliwell & Whiteman, 1997; Sies & Stahl, 1995). Ascorbic acid is not only a potent hydrophilic antioxidant; but is also essential in the regeneration of the oxidized form of α -tocopherol, a lipophilic potent free-radical scavenger (Roth, Manhart &

Wessner, 2004). Normal vitamin C levels have been found to be also essential for normal endothelial function, which is disrupted in severe sepsis (Wilson, 2009).

The requirement for vitamin C is increased in critical illness – as it is consumed following irreversible biochemical reactions (Berger, 2007). Increased oxidative stress overwhelms the available vitamin C supply, thereby causing a deficiency. Vitamin C concentration is reduced both in the plasma and endothelial cells in severe sepsis (Seno et al., 2004; Wilson, 2009). This will be discussed further in a subsequent section of this literature review.

1.3.2. Oxidative stress

Oxidative stress is a serious imbalance between free radicals and antioxidants (Halliwell, 1997). This occurs when there is increased free radical generation or decreased antioxidant protection with failure to prevent oxidative damage.

Oxidative stress can be measured. Reactive oxygen species have a very short half-life; and as such, obtaining a measure of oxidative stress is mainly dependent on the by-products of lipid, DNA and protein peroxidation (Berger & Chioléro, 2007). Thiobarbituric acid-reactive substances (TBARS) are markers of lipo-peroxidation which can be measured in human plasma (Goode et al., 1995; Takeda et al., 1984).

Intense intravascular oxidative stress occurs in critical illness – especially in severe sepsis; and it arises from the enormous production of free radicals in a non-regulated fashion under conditions of inadequate anti-oxidative defences (MacDonald, 2003; Galley et al., 1997; Doise et al., 2008). Uncontrolled oxidative stress has been associated with organ dysfunction and multi-organ failure, leading to increased morbidity and mortality (Biesalski & McGregor, 2007; Heyland et al., 2013).

Although critical illness has generally been associated with oxidative stress, patients that satisfy the systemic inflammatory response syndrome (SIRS) criteria, such as severe sepsis, have in particular been shown to have higher levels of oxidative stress and reduced total antioxidant capacity (Alonso de Vega et al., 2002; Ogilvie et al., 1991). Goode et al. (1995) demonstrated earlier that septic shock was associated with a marked decrease in circulating concentrations of antioxidant micronutrients; and that this was associated with increased TBARS.

These results have been supported by other studies (Andresen et al., 2008; Galley, Davies & Webster, 1996; Metnitz et al., 1999; Motoyama et al., 2003). The study by Goode et al. (1995), however, did not look at the relationship of oxidative stress to endothelial dysfunction, a phenomenon that has been understood to mediate the pathophysiology of organ dysfunction in severe sepsis (Edul et al., 2012). Furthermore, this study did not measure the vitamin C status, a potent antioxidant that promotes normal endothelial function in severe sepsis (Wilson, 2013). This is an area that, therefore, requires further exploration.

Organ dysfunction, which is a complication of severe sepsis, has been associated with further increases in oxidative stress (Himmelfarb et al., 2004), even more so in those with multi-organ failure (Motoyama et al., 2003). Ware et al. (2011) confirmed these findings and further demonstrated that increased oxidative stress is associated with renal, hepatic, and coagulation failure in critically ill patients with severe sepsis. However, their results did not show any significant difference in the levels of F2-isoprostanes, as markers of oxidative stress, between survivors and non-survivors.

Evidence shows that severe sepsis also predisposes the endothelial cell surface to oxidative stress. In a prospective, observational study in which endothelial cells were induced to produce ROS, and to analyse whether ROS production was correlated to severity of septic shock, Huet et al. (2007) demonstrated that plasma from septic shock patients caused naive human umbilical vein endothelial cells (HUVEC) to produce ROS. This study also showed that ROS production correlated with mortality and with the Sequential Organ Assessment Failure (SOFA) score. This illustrates that the endothelial cells play a significant role in the formation of ROS in severe sepsis – thereby suggesting that this could be part of the pathogenesis – leading to endothelial and microcirculatory dysfunction.

There is agreement in the literature concerning oxidative stress and its association with septic shock (Andresen et al., 2008; Berger & Chioléro, 2007; Wilson, 2013). However, while most studies showed a decrease in plasma vitamin C in septic shock, the concentration of other micronutrient antioxidants, such as vitamin E, has been inconsistently reported. Andresen et al. (2008) found that there was an associated low plasma vitamin C status in the patients. Surprisingly in this study, at baseline, the

vitamin E status of the patients was similar to that of the healthy control group. Also, while an increase in TBARS levels was associated with a decrease in vitamin C levels at 24 hours and 72 hours, and day 7, there was no significant change in the vitamin E plasma levels in this study (Andresen et al., 2008).

This study also showed that the plasma vitamin C in survivors only normalised at three months. The data from Andresen et al. suggest that vitamin C is one of the antioxidant micronutrients with high consumption levels during the first week of sepsis; and that these levels only normalise between one week from the onset of sepsis and three months. This could possibly be related to the participation of vitamin C in restoration of endothelial function in severe sepsis – rather than just being an antioxidant (Han et al., 2010; Tyml, Li & Wilson, 2008; Wilson, 2009; Wu, Wilson & Tyml, 2004; Wu et al., 2007; Zhou et al., 2012).

The other challenge that complicates the management of septic shock is stress hyperglycaemia.

1.4. Hyperglycaemia and its effects in critical illness

Clinically, critical illness is associated with a transient elevation of blood glucose levels in the absence of diabetes mellitus (including sepsis). This is termed stress hyperglycaemia (Dungan, 2009); and control of blood glucose levels in such clinical conditions is a challenge (Smith, 2000). Stress hyperglycaemia has been associated with poor clinical outcomes of critically ill patients. High maximum blood glucose levels during their stay in ICU have been correlated with increased morbidity, specifically infection, and mortality (Christiansen et al., 2004). Mortality rate in newly diagnosed hyperglycaemic patients was found to be six times higher than those with known diabetes, and therefore chronic, pre-existing hyperglycaemia (Umpierrez et al., 2002).

These findings have been supported by other research findings (Egi et al., 2008; Leonidou et al., 2007). Acute hyperglycaemia possibly influences clinical outcome by, but not only limited to, its contribution to oxidative stress and endothelial dysfunction. Control of blood glucose levels is therefore essential in critical care.

Van den Berghe et al., (2001) first demonstrated in their randomised controlled-trial of tight glucose control (4.4–6.2mmol/l) in adult ICU patients prevented excessive morbidity and mortality, compared with those tolerating stress hyperglycaemia – up to 12mmol/l. And several other studies have confirmed and supported this finding (Hirsch et al., 2002; Ellger & Van den Berghe, 2009; Van den Berghe et al., 2006; Van den Berghe, 2013). Another randomised, controlled study however favoured control of blood glucose levels between 7.8mmol/L and 10mmol/L (The NICE-SUGAR Study Investigators, 2009). It has thus been recommended in a review of such studies that blood glucose be maintained at levels as close to normal as possible, without evoking unacceptable fluctuations, hypoglycemia, and hypokalemia (Van den Berghe, 2009).

In critical care, the use of drugs such as steroids has also been shown to cause hyperglycaemia, because they reduce insulin sensitivity (Oyer, Shah & Bettenhausen, 2006); and consequently, the incidence of steroid-induced hyperglycaemia is high (Fong & Cheung, 2013).

Variability of glucose levels seems to be more deleterious than persistent hyperglycaemia. Repetitive postprandial fluctuations in glucose concentration have been shown to evoke monocyte adhesion to endothelial cells to a greater extent than that in diabetic rat models with stable hyperglycaemia. Suppression of such fluctuations efficiently suppressed leucocyte adhesion to the aortic endothelium (Azuma et al., 2006). However, this study group did not find any associated increase in the expression of mRNA for soluble vascular cell-adhesion molecule 1 (sVCAM-1) as a marker of endothelial dysfunction.

An *in vitro* study showed that endothelial cells exposed to intermittently elevated glucose levels could be associated with the overproduction of ROS, suggesting that *in vivo* glucose fluctuation may be involved in the development of oxidative stress and vascular injury (Quagliaro et al., 2003). This study group further reported that the exposure of endothelial cells to hyperglycaemic conditions, and even more so those with marked variations of glucose levels, increased the expression of several adhesion molecules, including soluble vascular cell adhesion molecule-1 and E-selectin (Quagliaro et al., 2005). The presence of sVCAM-1 and E-selectin provides

evidence for endothelial damage and activation from an insult (Chen et al., 2011; Cook-Mills, Marchese & Abdala-Valencia, 2011). However, these are only *in vivo* and animal-based studies. What have clinical studies shown on this topic?

In a case-control study, it was shown that acute hyperglycaemia in both type 1 diabetic and healthy individuals induces an inflammatory response that may be accompanied by oxidative stress (Gordin et al., 2008). Another clinical study reported decreased endothelial function using flow-mediated dilatation technique and increased urinary markers of oxidative stress in consistently high and fluctuating glycaemic conditions (Ceriello et al., 2008). Flow-mediated dilatation technique is designated as an endothelium-dependent process that reflects the relaxation of a conduit artery when exposed to increased flow and, thereby, increased shear stress (Moens et al., 2005). In humans, flow-mediated dilatation is usually assessed in large peripheral conduit arteries (brachial, radial, and femoral), imaged with adequately powered ultrasound equipment compatible with vascular 2D, colour flow, and pulse-wave Doppler techniques capable of recording arterial and Doppler measures for long periods continuously (Stout., 2009).

Further to this, an acute rise of hyperglycaemia was found to be independently associated with a simultaneous decrease in endothelial function and an increase in oxidative stress and inflammatory biomarkers in young adults with type 1 diabetes (Ceriello et al., 2010). Interestingly in this study, the administration of vitamin C – even in the presence of hyperglycaemia – restored endothelial function, and reduced oxidative stress and inflammatory biomarkers.

This finding was supported by another study, which demonstrated that ascorbic acid blocks the acute hyperglycaemic impairment of endothelial function in adolescents with type 1 diabetes (Hoffman, Dye & Bauer, 2012). It appears from these studies that hyperglycaemia induces an inflammatory response that is associated with oxidative stress, in a similar manner to that which occurs in acute severe injuries and sepsis. Stress hyperglycaemia may enhance the inflammatory response and hence increase oxidative stress, which may in turn influence endothelial dysfunction.

The challenge with these studies is that they were mainly done in participants who had pre-existing chronic hyperglycaemic conditions, such as diabetes; and these individuals could have had other vascular pathologies, which could possibly have influenced these results (Ceriello et al., 2010). However, in some studies (Gordin et al., 2008), healthy individuals were also studied; hence the results obtained could also reflect what would happen if blood glucose levels were acutely elevated in critically ill patients with stress hyperglycaemia.

Having discussed the adverse effects of stress hyperglycaemia in critical illness, and highlighted that these effects could be attenuated by vitamin C, it is important to note that there is an interesting relationship between vitamin C and glucose. The mechanisms of transport of each across the cell membrane are competitive, and this has clinical implications for patients with severe sepsis and hyperglycaemia, as described below.

Vitamin C exists in endothelial cells mainly in the form of ascorbic acid (ascorbate), which is transported into the cell through a specific sodium-dependent vitamin C transporter 2 (SVCT2) (Seno et al., 2004). This is in contrast to the exterior of the cell where vitamin C mainly exists in its oxidised form, DHA. DHA is structurally similar to glucose. Rumsey et al. (1997) first illustrated that glucose transporter isoforms GLUT1 and GLUT3 also facilitate the transport of dehydroascorbic acid into the cell. After being transported into the cells, dehydroascorbic acid is quickly reduced to ascorbic acid (Wilson, 2009).

Usually, the endothelial cells tend to retain ascorbic acid (Davis et al., 2006; Wilson, 2005; Wu et al., 2007) so that the concentrations are higher than in the extracellular fluid; and it is only released to the extracellular fluid in a regulated fashion (Wilson, 2009) using calcium-dependent mechanisms (Davis et al., 2006).

In hyperglycaemic conditions, glucose competitively inhibits the uptake of dehydroascorbic acid by the endothelial cells; and so, hyperglycaemia may mimic vitamin C deficiency. In normal physiological conditions in healthy individuals, the expected glucose-to-vitamin C ratio has been estimated to be 88:1 (Price, Price & Reynolds, 2001). However, this ratio may rise further in acute hyperglycaemic

conditions. Furthermore, hyperglycaemia is known to increase the urinary loss of vitamin C (Will & Byers, 1996). Ascorbate deficiency mediated by acute hyperglycaemia in endothelial cells has been ascribed to the impairment of endothelium-dependent vasodilation in healthy human subjects – an effect which can be reversed by the acute administration of vitamin C (Price, Price & Reynolds, 2001; Wilson & Wu, 2012; Williams SB et al., 1998; Biesalski & McGregor 2007).

Thus, the plasma-vitamin C to glucose ratio also seems important for adequate vitamin C bioactivity. Possibly an increase in the concentration of vitamin C would correct hyperglycaemia-induced inhibition of dehydroascorbic acid uptake by the endothelial cells, and improve vaso-responsiveness and restore normal endothelial function. Hyperglycaemia-induced vitamin C deficiency is exacerbated by decreased uptake and consumption of vitamin C, as a consequence of the inflammatory response as is described below.

In a laboratory study using cultured human umbilical-vein endothelial cells, it was demonstrated that inflammatory molecules, such as TNF- α and IL-1 β , significantly suppressed the uptake of ascorbic acid through the SVCT2 in a dose- and time-dependent manner (Seno et al., 2004). This implies that low vitamin C status in plasma and endothelial cells in septic shock with hyperglycaemia would result from the following mechanisms: Firstly, due to losses by oxidative stress where ROS may oxidize ascorbate to DHA, and then oxidize the latter irreversibly; and secondly, through the increased urinary excretion that occurs in hyperglycaemic states (Will & Byers, 1996). Thirdly, the inhibition of SVC2 transport of ascorbic acid by inflammatory molecules; and lastly, by the effect of competitive inhibition of dehydroascorbic acid transport by GLUT1 and GLUT3 in hyperglycaemic conditions, where the glucose-to-vitamin C ratio is significantly raised (Wilson, 2009).

It has further been illustrated in an *in vitro* study that ascorbate blocks the stimulation of ROS production through many pathways – including NADPH oxidase activity in microvascular endothelial cells (Wu et al., 2007). It is also well-known that vitamin C supplementation results in the mitigation of the inflammatory response in septic animal models (Fisher et al., 2011). These properties of vitamin C, in addition to its

ability to reverse hyperglycaemia-induced endothelial dysfunction, would suggest that supplementation in septic shock might improve outcome. It would be of interest, therefore, to measure and investigate the ratio of glucose-to-vitamin C in septic shock, and its relationship to oxidative stress and markers of endothelial dysfunction.

Apart from oxidative stress and hyperglycaemia, the microcirculation has recently been recognised as being of primary importance in septic shock, where the normal endothelial function is deranged (Kanoore et al., 2013; De Backer et al., 2013).

1.5. Endothelial function

1.5.1. Endothelial function under normal physiological conditions

Before looking at the microcirculatory dysfunction associated with sepsis, it is important to briefly explain how the normal endothelium functions.

Although only a simple monolayer, the healthy endothelium has been described as optimally placed and able to respond to physical and chemical signals by the production of a wide range of factors that regulate vascular tone, cellular adhesion, thromboresistance, smooth muscle cell proliferation, and vessel-wall inflammation (Deanfield, Halcox & Rabelink, 2007). Through the production and release of several vasoactive molecules that relax or constrict the vessel, and through its response to the circulating vasoactive mediators, such as bradykinin and thrombin, the endothelium is able to affect vascular tone (Deanfield, Halcox & Rabelink, 2007). In effect, these functions have a direct role in the balance of tissue-oxygen supply and metabolic demand through the regulation of vessel tone; and they are involved in the remodelling of vascular structure and long-term organ perfusion (Stamler et al., 2003).

In normal physiology, nitric oxide has been proven to be the main relaxant factor in blood vessels (Furchgott & Zawadzki, 1980). It is derived through the conversion of L-arginine to citrulline, by the action of the endothelial nitric oxide synthase system (eNOS), in the presence of co-factors such as tetrahydrobiopterin (BH₄) (Förstermann & Münzel, 2006). The eNOS is involved in the production of nitric

oxide; and under normal physiological conditions, it is responsible for normal endothelial functions (Tyml, Li & Wilson, 2008).

However, these normal processes are disrupted in severe sepsis. Endothelial dysfunction has been closely associated with the pathophysiology of the progression of sepsis to septic shock that results in multi-organ failure. Actually, the definition of septic shock in itself describes a derangement in the endothelium, where severe sepsis is complicated by acute circulatory failure, which is characterised by persistent arterial hypotension – despite adequate fluid resuscitation, or by tissue hypoperfusion – that is manifested by a raised lactate concentration, and which is unexplained by any other causes (Dellinger et al., 2008).

1.5.2. Endothelial dysfunction in sepsis

Endothelial dysfunction in sepsis is characterised by a disruption of the endothelial barrier properties (Seno, 2004; Wilson, 2009); as well as by decreased endothelial reactivity to vasoconstrictive and vasodilatory substances (De Backer et al., 2013). These are mediated by increased leukocyte and platelet aggregation, and an increase in inducible nitric oxide synthase (iNOS) expression, which is the pathological nitric oxide synthase pathway that can exert a negative feedback on the endothelial nitric oxide synthase expression (Boisramé-Helms et al., 2013). It has been established, through experimental models of sepsis, that septic insults to the endothelial cell, stimulate NADPH oxidase and iNOS activity via cytokines (Wu, Wilson & Tyml, 2003; Li et al., 2005). Inducible nitric oxide synthase is also responsible for the generation of nitric oxide in amounts 1000-fold greater than constitutive amounts in basal endothelial function.

The consequences of this progression include a severely disrupted ability of the vascular smooth muscle to respond appropriately to vasoconstrictors, resulting in the refractory shock, which often occurs in severe sepsis. Furthermore, NADPH oxidase elevates production of ROS by synthesizing superoxide inside endothelial cells, and setting off free-radical chain reactions (Li et al., 2005). These free radicals cause injury to the endothelium, and play a role in the impairment of capillary blood flow and capillary barrier functions (Donati et al., 2013). This causes the microvasculature

to become leaky to fluid and renders the maintenance of adequate blood pressure by the provision of intravenous (IV) fluid challenging.

One of the other mechanisms associated with microcirculatory dysfunction in literature; is activation of coagulation. Using *in vitro* controlled experiment models, it was illustrated that normal neutrophils exposed to plasma from patients with septic shock demonstrated a significant increase in aggregation and endothelial cell adherence with associated decreases in neutrophil rolling velocity (Kirschenbaum et al., 2004). These changes were significantly enhanced in the presence of platelets and significantly attenuated in the presence of serum, which is fibrinogen depleted. Microvascular perfusion at the surface of these cell cultures was altered more with septic plasma than with septic serum, and especially in the presence of platelets.

Another study of skeletal muscle microcirculation in septic wild male mice supported these findings and further demonstrated that sepsis increases platelet aggregation, fibrin deposition, and the propensity for thrombosis in capillaries and that this process is P-selectin dependent (Secor et al., 2010). These data highlight the role of coagulation factors, leucocyte adhesion and platelets in microvascular alterations, even in the absence of overt thrombotic events.

Marechal et al. (2008) in their experiments with septic rats found that, apart from the hyporeactivity of vessels to vasoactive agents, there were associated microvascular derangements. There was decreased capillary density and significant increases in non-perfused and intermittently-perfused capillaries in the small intestine muscularis layer of septic rats, compared with the controls. These changes were associated with degradation of the glycocalyx. The glycocalyx is the gel-like layer covering the endothelial surface; and there has been increased research on this area in recent years (Woodcock & Woodcock, 2012). The glycocalyx is very sensitive to oxidative stress and increased inflammatory mediators; and degradation of the glycocalyx is, therefore, prominent in sepsis (Marechal et al., 2008). It has also been demonstrated in other studies that LPS-elicited plasma hyaluronan release and a reduction in endothelial surface thickness is indicative of glycocalyx degradation (Henry & Duling, 2000; Nieuwdorp et al., 2009).

A recent study in acutely ill patients by Ince's group showed that patients with sepsis had increased degradation of the glycocalyx; and this was associated with microvascular blood flow derangements (Donati et al., 2013). Increased production of inflammatory mediators and ROS also influence oxidative stress-induced disruption of tight junctions by mechanisms such as protein modification such as thiol oxidation, phosphorylation, nitration and carbonylation (Rao, 2008). Heterogeneous capillary blood flow has been observed in different tissues, where tissue oxygenation was impaired; and multiple organ dysfunction and failure ensued (Ince, 2005).

1.5.2.1. Soluble vascular cell adhesion molecule-1 (sVCAM-1) and E-selectin as biomarkers of endothelial dysfunction

There are some biomarkers associated with endothelial dysfunction and damage in sepsis. These biomarkers are expressed when the endothelium is activated or damaged – not only during sepsis – but also during systemic inflammation of any cause, and by biochemical alterations, such as shear stress and hydrostatic pressure (Reinhart et al., 2002). Endothelial activation is mediated by cytokines (for example interleukin-1 (IL-1) and TNF- α), proteases, growth factors and vasoactive compounds (Hack & Zeerleder, 2001).

The biomarkers of endothelial dysfunction include E-selectin, soluble vascular adhesion molecule 1 (sVCAM-1), platelet-derived growth factor (PDGF), endothelin (ET), and intercellular adhesion molecule-1 (ICAM-1) (Reinhart et al., 2002). E-selectin and sVCAM-1 will be briefly discussed because of their relevance to this study.

E-selectin is a glycoprotein that mediates rolling adhesion, the first step in leukocyte adhesion onto the endothelium (Reinhart et al., 2002). E-selectin is unique to the endothelium; and, in most vessels, de novo synthesis and expression can be induced within two hours and peaks at about six hours, in response to inflammatory stimuli or disturbed blood flow (Kansas, 1996; Sipkins et al., 2005). Studies have illustrated that E-selectin is an early marker for sepsis and the systemic inflammatory response, regardless of the cause, following stimulation by endotoxin and pro-inflammatory cytokines (Cummings et al., 1997; Quagliaro et al., 2005). But it may remain up-

regulated in the endothelium in areas of chronic inflammation (Keelan et al., 1994; Picker et al., 1991). It is also clear that E-selectin is expressed in viable endothelial cells, mediates apoptosis, and that necrosis does not participate in its release (Harrington et al., 2006; Leeuwenberg et al., 1992; Pigott et al., 1992).

E-selectin has been associated with angiogenesis (Koch et al., 1995); and its expression has been detected in the skin and parts of bone-marrow microvasculature (Sipkins et al., 2005). E-selectin has been shown to be involved in tumour growth and metastasis in bone tissue (Läubli & Borsig, 2010). This would possibly mean that the expression of E-selectin would be enhanced in both inflammation and repair of the micro-vasculature. In fact, a recent study has confirmed that E-selectin is a marker of endothelial proliferation (Smadja, Mulliken & Bischoff, 2012). However, concerning organ failure and the risk of mortality, there has been controversy concerning the relationship between plasma E-selectin concentration and these variables (Cummings et al., 1997; Shapiro et al., 2010). Soriano et al. (2005), in their study, found that all markers of cell activation and inflammation (which stimulate E-selectin expression), were significantly higher among survivors than non-survivors of sepsis – with the exception of nitric oxide, which was lower.

These data support the hypothesis that an early increase, not decrease in inflammatory response, can be associated with improved survival rate (Soriano et al., 2005). Interestingly, these authors in this same study demonstrated that there was a negative correlation between the markers of inflammation and the SOFA score. This suggests that lower levels of plasma E-selectin could be expected with worsening organ failure. It is not surprising, therefore, that E-selectin has been found to be a less reliable marker of organ dysfunction and mortality than other endothelial markers, such as soluble VCAM-1 and soluble fms-like tyrosine kinase 1 (sFlt-1) (Skibsted et al., 2013).

Soluble VCAM-1 participates in the adhesion of leukocytes to the endothelium at the site of inflammation (Paulus, Jennewein & Zacharowski, 2011). Unlike E-selectin, sVCAM-1 is expressed on leukocytes and a variety of other cell types, in addition to the endothelium (Reinhart et al., 2002). Soluble VCAM-1 has mechanical functions and is also involved in the intercellular signal transduction of polymorphonuclear

leukocytes (Paulus, Jennewein & Zacharowski, 2011). This molecule mediates leukocyte activation events that may lead to tissue damage by the release of lysosomal enzymes and the production of ROS (Reinhart et al., 2002). Increased plasma levels of sVCAM-1 would therefore be expected in both conditions of increased cellular damage and increased oxidative stress. A persistent increase in plasma sVCAM-1 was observed in septic patients with persistent multi-organ failure (Whalen et al., 2000).

Endothelial dysfunction subsequently gives rise to the microcirculatory dysfunction that has been observed in both animal and human studies.

1.5.3. Microcirculatory alterations, the mechanisms and consequences in sepsis

It has been shown through many clinical studies by Can Ince's group that microcirculatory dysfunction is the motor for sepsis-induced organ dysfunction and failure (Gomez et al., 2014; Ince, 2005; Piagnerelli, Ince & Dubin, 2012).

Most studies in animal models of sepsis have reported deranged capillary perfusion in different organs. Farquhar et al. (1996) in a classical randomised controlled study reported decreased perfused capillary density of the small bowel in a normotensive rat caecal ligation and perforation (CLP) model of sepsis. Although limited by a small number of analysed animals (six) in each group, this study described deranged microcirculation that occurred despite maintenance of mean arterial pressure by intravenous fluid administration – a model that would explain organ hypo-perfusion in severely septic patients – even when blood pressure is within the normal range. Similar results were found by Ellis et al. (2002).

In another study in male pigs – using a modified orthogonal polarization spectral device to study the microcirculation, it was shown that septic shock caused a markedly decreased functional microvascular density of the sublingual and gut regions (Verdant et al., 2009). The weakness of this study was that there was no use of vasopressors, as is the case in human septic shock. Furthermore, this study had an imbalance between the sham and intervention animals. It also had small sample sizes – of three and seven – in sham and septic animals, respectively, reducing the power of the study. Additionally, there could be inter-species differences in the vascular

response in sepsis between pigs and humans; and so this may not be directly translated to humans. Despite these limitations, the study had a key finding that the sublingual circulation, just like the bowel microcirculation, could be of use in the study of microcirculation in severe sepsis. This technique is now used in human microcirculation studies.

A more recent study showed, for the first time, the possible mechanisms associated with microcirculatory dysfunction (Secor et al., 2010). This study utilised a fluid resuscitated model of polymicrobial sepsis in male wild type mice injected with faeces into the peritoneum (FIP). The mice were eNOS knockout (eNOS^{-/-}), iNOS knockout (iNOS^{-/-}), and gp91phox (a subunit of NADPH oxidase) knockout (gp91phox^{-/-}). The results showed, for the first time, that sepsis increases platelet aggregation, fibrin deposition, and the propensity for thrombosis in capillaries – and that capillary flow stoppage requires platelets, P-selectin, and coagulation activation.

The same authors further illustrated in this study that capillary platelet adhesion can be prevented or reversed by gp91phox and iNOS deficiencies, ascorbate, and local BH4. This study is important, since it describes the possible mechanisms associated with microvascular dysfunction in severe sepsis; and hence, it offers some insight into the exploration of treatment targets. Interestingly enough, the study suggests that the microvascular dysfunction could be prevented, and even reversed. It also suggests that platelet adhesion to the endothelium that leads to capillary blood flow impairment is enhanced by ROS, which promotes the P-selectin expression, and is dependent on NADPH oxidase.

Have human clinical studies shown similar effects on the microcirculation in severe sepsis? It has been shown using an orthogonal polarization spectral imaging technique that, patients with severe sepsis have decreased functional microvascular density than healthy volunteers and non-septic critically ill patients (De Backer et al., 2002). These results confirmed, and were supported by other human Doppler techniques (Hernandez, Bruhn & Ince, 2013; Edul et al., 2012; Sair et al., 2001) or plethysmography (Kirschenbaum et al., 2000; Sair et al., 2001; Nevriere et al., 1996; Young & Cameron, 1995).

Non-survivors of severe sepsis have been shown to have worse microvascular dysfunction than survivors (De Backer et al., 2002). This would infer that, factors that lead to microcirculatory dysfunction such as oxidative stress, activation of platelet plugging, and turning on of iNOS (Secor et al., 2010; Tymł, Li & Wilson, 2008) are more pronounced in these patients. On the other hand, this could also mean that known factors that prevent or reverse these effects, such as vitamin C and BH₄ (Armour et al., 2001; Wilson, 2009; Wu, Wilson & Tymł, 2003; Tymł, Li & Wilson, 2008a) are either inhibited or they are in lower concentrations in non-survivors than they are in survivors.

None of the human studies on the microcirculation in septic shock presented here was a randomised control trial. Moreover, these studies did not concentrate on the mechanisms that could give insight into treatment options. Nevertheless, these studies confirm that human sepsis is associated with microcirculatory dysfunction, which leads to multi-organ dysfunction and failure. The evidence further suggests that the microcirculatory pathological changes are more pronounced in non-survivors than in survivors; and they are a strong predictor of outcome (De Backer et al., 2013a; Trzeciak et al., 2007).

There is hope, however; and it lies in the fact that the microcirculatory lesion associated with septic shock in humans is reversible. It has been shown that local application of acetylcholine was able to reverse the sepsis-induced microvascular blood flow alterations (De Backer et al., 2002), confirming previous findings in the experimental models of septic shock in animals (Secor et al., 2010; Wilson, 2009; Wu, Tymł & Wilson, 2002).

With this evidence, the hypothesis that targeting the microcirculation, distinct from the macrocirculation, could potentially improve organ failure in sepsis (Trzeciak et al., 2008), and be the way forward in reducing the morbidity and the mortality associated with septic shock. It should be considered, however, that microcirculatory alterations are not the sole mechanism contributing to the organ dysfunction, because cellular metabolic alteration – including mitochondrial dysfunction – also contributes to the condition (De Backer et al., 2013).

On the other hand, addressing the microcirculatory dysfunction could still be a step forward in addressing the morbidity, and possibly mortality, associated with septic shock. This could partly be achieved through addressing sepsis-induced oxidative stress – by supplementation with a substance known to promote normal endothelial dysfunction – and one which has the potential to prevent and reverse microvascular dysfunction, such as vitamin C.

1.6. The role of vitamin C in severe sepsis

Recently, there has been strong evidence forthcoming, suggesting that the supplementation of vitamin C in sepsis would be one of the potential therapeutic strategies in the management thereof (Wilson, 2013). The next section reviews the micronutrient vitamin C and the available evidence of its various roles in the treatment of sepsis.

Ascorbate has been shown to influence survival in mouse models of sepsis. Gaut et al. (2006), showed that that ascorbate-deficient mice had an increased mortality following infection with *Klebsiella pneumoniae*, a gram-negative bacterium that also causes sepsis in humans (Heyland et al., 2013), compared to mice on vitamin C supplementation. Prior to this, Wu et al. (2004) found that an intravenous bolus of 200mg/kg of ascorbate before introduction of infection through caecal ligation and puncture (CLP) in wild type mice dramatically increased the survival of the septic mice at 24 h post-CLP.

This study also illustrated that an ascorbate bolus had beneficial systemic effects, including the prevention of oxidative stress, impaired pressor response to angiotensin II, and the maintenance of baseline arterial pressure. For the first time, this study group also showed that ascorbate inhibits the expression of iNOS mRNA induction in arterioles, the overproduction of nitrites/nitrates, and impaired arteriolar vasoconstriction response to angiotensin II. Ascorbate was also shown to have these benefits – even after a three-hour delay in these animals – a phenomenon that could be beneficial in the late phases of sepsis in humans. This study had the advantage that, even though it did not include antibiotic therapy, it included testing of the response of the arterioles to vasoconstrictors, following ascorbate infusion, a response which is normally deranged in human septic shock. However, this study did

not incorporate intravenous fluid administration and antibiotics, as is usual in the treatment of human sepsis.

Vitamin C has also been shown to inhibit and reverse the capillary blood flow impairment that occurs in sepsis, by inhibiting the NADPH oxidase activity, which plays a major role in this pathophysiology (Tyml, Li & Wilson, 2008). In addition, ascorbate was found to prevent or reverse sepsis-induced deficit in conducted arteriolar vasoconstriction and sepsis-induced increase in neuronal nitric oxide synthase (nNOS) enzymatic activity (Mckinnon, Lidington & Tyml, 2007).

Another existing clinical challenge associated with severe sepsis is sepsis-induced lung injury. Fisher et al. (2011) conducted a laboratory study in wild type mice in which acute lung injury had been induced. It was discovered that mice without intervention demised within 28 hours of administration of LPS. On the other hand, in the intervention group that received vitamin C supplementation, up to 75% survived at 72 hours. Moreover, while there was some loss of capillary barrier function, exuberant pulmonary inflammation, and extensive microthrombus formation in the group without vitamin C supplementation, the group that received vitamin C supplementation had preserved lung architecture and barrier function, while showing attenuated pro-inflammatory chemokine expression and microvascular thrombosis.

These results show that vitamin C, quite apart from having antioxidant properties, has other beneficial properties in the treatment of sepsis; hence, it has the potential to reduce morbidity and mortality if these properties were to be realised in human septic shock. Although animal data, whatever the species, cannot be applied directly to humans, these experiments do give us some understanding of the mechanisms of these effects, as also seen in humans (Berger, 2005).

There have been few clinical studies in humans that have looked at vitamin C and critical illness. Long et al. (2003) found that only intravenous supplementation of at least 3000mg for at least 48 hours can restore the levels to normal. In another randomised controlled trial of post-operative patients, overnight intravenous administration of vitamin C, depending on the deficit, was shown to normalise the plasma vitamin C levels to either normal or just above normal (Rümelin et al., 2005).

These two studies, however, did not report on the clinical impact of this replenishment.

High dose vitamin C supplementation has been associated with improved clinical outcome in burns patients. Tanaka et al. (2000), in a randomised prospective study involving 37 patients with severe burns, supplemented high dose vitamin C at a dose of 66mg/kg in the first 24 hours of admission, and followed the patients for 7 days. Despite the small sample size of the study, it was shown that adjuvant high vitamin C supplementation reduced serum malondialdehyde levels, as markers of oxidative stress; and it reduced the amount of intravenous fluid required for resuscitation, reduced wound oedema; and it reduced the severity of respiratory dysfunction. This study is relevant because severely burnt patients exhibit characteristics similar to those of severe sepsis, where there is an increased level of oxidative stress and the presence of systemic inflammatory-response syndrome.

Another randomised controlled trial, this time in critically ill surgical patients, showed that administration of α -tocopherol and ascorbic acid reduced the incidence of organ failure and shortened the ICU length of stay in this cohort (Nathens et al., 2002). However, this study did not look at the clinical effects of pure vitamin C alone. A similar randomised controlled, double-blinded trial by Berger et al. (2008) showed that antioxidant supplementation, including 1.1g of vitamin C, significantly reduced the inflammatory response in cardiac surgery and trauma patients, a property that would surely be beneficial in severe sepsis.

Vitamin C supplementation has also been associated with a decrease in organ dysfunction. Giladi et al. (2011), showed that supplementation with 1000mg vitamin C intravenously, 8 hourly, alongside α -tocopherol 1000 IU 8 hourly and selenium 200 mcg daily for seven days, was associated with a reduction in respiratory failure, reduced ventilator-dependence, and a marked decrease in abdominal wall complications. It is not known, however, whether these results would be reproducible with pure vitamin C alone.

1.7. Conclusion

Septic shock is one of the most common disease processes in critical illness, and despite advances in its management over the years, it is associated with mortality of more than 30% in most settings (Kethireddy & Kumar, 2012; Labelle et al., 2012; Roman-Marchant et al., 2004). There is intense intravascular oxidative stress, which arises from the enormous production of free radicals in a non-regulated fashion, under conditions of inadequate antioxidative defenses (Macdonald, 2003; Galley et al., 1997; Doise et al., 2008). The requirement for vitamin C is, therefore, increased in critical illness, partly due to the need for replenishing antioxidant losses following irreversible biochemical reactions (Berger 2006). Not only is vitamin C found in low plasma concentrations; but it is also depleted in the endothelial cells in cases of severe sepsis (Wilson, 2009; Seno et al., 2004).

The microcirculation has recently been recognised as the centre of several pathophysiological processes in septic shock (Kanoore et al., 2013), where normal endothelial function is deranged. It has recently been established that the endothelial dysfunction is mediated by the destruction of the glycocalyx, which is very sensitive to oxidative stress and high levels of inflammatory mediators (Donati et al., 2013).

Another consideration is that the uncontrolled hyperglycaemia typical in sepsis and critical illness is one of the factors said to be responsible for the depletion of ascorbic acid in the endothelial cells, due to competitive inhibition of glucose on glucose transporters (Wilson 2009). Thus, the plasma vitamin C to glucose ratio seems important for adequate vitamin C bioactivity; but this has not been investigated well in cases of septic shock.

Recently, there has been strong evidence suggesting that the supplementation of vitamin C in sepsis could be one of the potential therapeutic strategies in its management (Wilson, 2013). Vitamin C has the potential to replenish plasma vitamin C levels, to reduce oxidative stress, to prevent and reverse microcirculatory dysfunction, and to prevent, and even reverse, the adverse effects of hyperglycaemia, and also to reduce the morbidity and mortality associated with septic shock (De Backer et al., 2013a; Giladi et al., 2011; Nathens et al., 2002; Tanaka et al., 2000; Wilson & Wu, 2012; Biesalski & McGregor 2007).

Before exploring the effects of high dose vitamin C in septic shock in a South African setting, it is of value to put forward the following questions:

In a population of critically ill patients with septic shock requiring inotropes,

1. What is the plasma vitamin C status?
2. What is the oxidative status of these patients?
3. What are the levels of endothelial dysfunction markers in this patient group?
4. What is the glucose-to-vitamin C ratio in this patient group?
5. What are the associations of the above parameters with each other, the SOFA score, intravenous fluid requirements, and the inotrope requirement?

With these questions in mind, this study was embarked upon.

CHAPTER 2: PROJECT AIM AND OBJECTIVES

2.1. Aim

To investigate the vitamin C status, the oxidative stress status, hyperglycaemia and endothelial function in critically ill patients with septic shock on inotropes.

2.2. Specific objectives

In a population of critically ill patients with septic shock requiring inotropes:

- To measure the plasma vitamin C status;
- To measure the plasma markers of oxidative stress;
- To investigate hyperglycaemia and the plasma glucose-to-vitamin C ratio;
- To measure the biomarkers of endothelial dysfunction;
- Investigate the difference in the above parameters between survivors and non-survivors;
- To investigate the associations of the above parameters with each other, the SOFA score, intravenous fluid requirements, and the inotrope requirement.

2.3. Hypotheses

H_0 = Critically ill patients with septic shock have normal vitamin C status, no indication of oxidative stress, and no indication of endothelial dysfunction.

H_A = Critically ill patients with septic shock have low vitamin C status, high concentrations of indicators of oxidative stress and indicators of endothelial dysfunction.

CHAPTER 3: MATERIALS AND METHODS

3.1. Study design

This is a prospective, cross-sectional study.

3.2. Study population and location

Participants included in the study were patients admitted to the multidisciplinary Intensive Care Units (ICU) at Groote Schuur Hospital, which is a tertiary-level facility, and an academic teaching hospital in Cape Town, South Africa.

3.3. Inclusion and exclusion criteria

3.3.1. Inclusion criteria

- Adult patients (18 years and above)
- Admitted to the multidisciplinary ICU with septic shock, requiring inotropic support expected to continue for at least 24 hours

3.3.2. Exclusion criteria

- BMI of less than 18kg/m²
- Patients who – in the judgement of the intensivist – were not expected to survive more than 24 hours, despite maximal organ support
- Patients who were on parenteral nutrition support at the time of screening for the study
- Pregnant patients
- Patients with gastrointestinal fistulas or other considerable exudative losses
- Patients requiring renal dialysis at the time of study screening
- Patients having had vitamin C supplementation of more than 100mg/day during the 7 days prior to screening

3.4. Sample-size calculation

Using an alpha error of 5% and power of 80%, the sample size required to detect the prevalence of deficient vitamin C status, was calculated as follows:

Prevalence of deficiency	40%	60%	80%
Required sample size	23	18	15

Since the expected prevalence of vitamin C deficiency in South African ICUs is not known, the study enrolled 25 patients – with the aim of detecting at least a 40% prevalence of vitamin C deficiency.

3.5. Ethical considerations and approval

3.5.1. Ethics and institutional approval

The study was approved by the Human Research Ethics Committee of the Faculty of Health Sciences, University of Cape Town (Reference number UCT FHS HREC 528/2011), and the medical superintendent for research of Groote Schuur Hospital. No subject was enrolled in the study until the protocol and subject information had been approved in writing by the Human Research Ethics Committee.

3.5.2. Informed consent

The study was performed in accordance with the principles of the Declaration of the Helsinki (2008), Good Clinical Practice (GCP) guidelines, and the laws of South Africa.

Since the study population consisted of critically ill adults in ICU, the usual process of prior informed consent was not possible, because the patients were not competent to provide informed consent on their own behalf at the time of enrolment. The International Conference for Harmonisation (ICH), Good Clinical Practice (GCP) guidelines (4.8.15) states that: “In emergency situations, when prior consent of the subject is not possible, the consent of the subject's legally acceptable representative, if present, should be requested. When prior consent of the subject is not possible, and the subject’s legally acceptable representative is not available, enrolment of the subject should require measures described in the protocol and/or elsewhere, with documented approval/favourable opinion by the IRB/IEC, to protect the rights, safety

and wellbeing of the subject, and to ensure compliance with applicable regulatory requirements. The subject, or the subject's legally acceptable representative should be informed about the trial as soon as possible; and consent to continue and other consent as appropriate, should be requested.”

The South African GCP guidelines (2.3.6.1) on research in critical care state that; “Characteristic features of intensive care research are the difficulties in communicating with patients receiving ventilator assistance, and the impairment of cognition in heavily sedated individuals. Whenever possible, information regarding intensive care research should be obtained from potential participants before their admission to that care”.

Furthermore, regarding research involving unconscious patients, the South African GCP guidelines (2.3.6.5) state that: “The distinguishing feature of research with unconscious persons is that, because of their incapacity for cognition or communication, it is impossible for them to be informed about the research, or for a researcher to determine their wishes about it. Consent to participation in research by an unconscious person must be given by others, including relevant statutory authorities, on that person's behalf. Because of their extreme vulnerability, unconscious persons should be excluded from all but minimally invasive observational research. When research procedure precludes conformity to the principle of consent, and neither the prospective participant, nor the participant's representative, is able to give consent in advance, a research ethics committee may approve a research project without prior consent, if it is satisfied that:

- Inclusion in the research project is not contrary to the interest of the patient;
- The research is intended to be therapeutic, and the research intervention poses no more of a risk than that inherent in the patient's condition and alternative methods of treatment;
- The research is based on valid scientific hypotheses, which support a reasonable possibility of benefit over standard care; and
- As soon as reasonably possible, the participant and the participant’s relatives or legal representative will be informed of the participant’s inclusion in the research, and will be advised of their right to withdraw from the research, without any reduction in [the] quality of care.

In the case of research proposals in which it is impractical to obtain consent before including in the research, a participant who is highly dependent on medical care, a research ethics committee must then be satisfied that:

- Adequate provision will be made for informing patients and their relatives about the research, to ensure that stress and other emotional factors do not impair their understanding of it; and
- The dependency of patients and their relatives on the medical personnel providing treatment does not affect any decision to participate”.

Due to the observational nature of the study, and the minimal risk to study the participants, we were permitted to utilise a deferred informed consent procedure (see Appendix B1). Patients were enrolled in the study when they met the eligibility criteria. All blood samples and data collected were stored until the patient was well enough to give written informed consent for the use of such data. If the patient declined participation, all data and derived data were excluded from the study, and destroyed. In the case where a patient died before deferred written informed consent could be obtained, informed consent was waived; and we were permitted to utilize the data.

The inclusion of patients who did not survive septic shock was important for the understanding of the factors associated with mortality in septic shock in the study.

3.5.3. Confidentiality and anonymity

To ensure the participant’s privacy and confidentiality, only authorised individuals working in the study had access to the individual information collected. The data from each individual patient were uniquely coded, so as not to identify the individual. The blood samples and the data generated in the study were also coded for analysis, in order to protect the patients’ identities.

3.5.4. Participants’ rights and autonomy

Participants had the right to withdraw, or to withdraw their data from the study, at any stage. The participants were protected by an insurance policy held by the University of Cape Town – in the unlikely event that they suffered a direct injury related to the research study.

3.6. Participant recruitment

Critically ill, septic patients in the ICU, or those being admitted to the ICU, were screened for study inclusion, as soon as they developed the need for inotropic support. The patients were recruited for the study within a 12-hour window from commencement of their treatment with inotropes. Those patients who satisfied the inclusion criteria were enrolled by a member of the research team. The patients were not re-enrolled for subsequent septic shock episodes, or for subsequent periods, where inotropes were required; but instead, they continued, according to the study assessment schedule.

3.7. Data collection

The data were collected from all the patients who were recruited, since they satisfied the inclusion criteria. A designated member of the study team collected the data from the participants. Clinical and demographic data were collected from the patients' clinical records and from their charts during their admission. Blood sampling was done at baseline on enrolment – then daily – until inotrope cessation, and thereafter, at day seven, following the cessation of inotropic support. Laboratory analysis was done in the Redox laboratory of the Human Biology Department, Faculty of Health Sciences, at the University of Cape Town.

3.7.1. Participant demographics

Demographic data were recorded for all the participants of the study; and this included:

- Age
- Gender
- Dates of hospital and intensive care (ICU) admission
- Main diagnosis
- Acute Physiology and Chronic Health Evaluation (APACHE) II score

3.7.2. Anthropometric measures

Following enrolment, the BMI of all the patients was estimated by a clinical dietician, according to the usual clinical practice. The recumbent length of the patient was measured, using a rigid length measuring stick, with the patient lying supine,

and with the bed in the flat position. The estimated body weight was then derived from the estimated BMI and actual recumbent length.

3.7.3. Clinical data

During their stay in ICU, patients were followed up daily commencing with the day of study inclusion (day 0) until ICU discharge or death (whichever came first); and during this time, the following clinical data were recorded from routine clinical charts and the medical records:

- Daily lowest and highest mean arterial blood pressure
- Daily mean blood glucose and ranges
 - a) Inotrope requirement, which comprised: the number of days on inotropes;
 - b) The total amount of inotropes used per day and per kg body mass;
 - c) Inotrope-free days at day seven of the study period.
- Daily fluid intake, output from all sources, and calculated fluid balance;
- Calculated vitamin C intake from all enteral feeds and parenteral sources;
- Daily Sequential Organ Failure Assessment (SOFA) score. The score is based on six different scores, one each for the: respiratory, cardiovascular, hepatic, coagulation, renal and neurological systems. Each organ score has a maximum worst score of 4, and a best score of 0. A final score is attained by adding the scores for all the organs, as seen in Table 3.1.

Table 3.1: SOFA Score

Organ System	Score				
	0	1	2	3	4
Respiratory: PaO ₂ /FiO ₂	>400	≤400	≤300	≤200	≤100
Renal: Creatinine (μmol/l)	≤110	110-170	171-299	300-440;urine output ≤500 ml/day	440; urine output <200 ml/day
Hepatic: bilirubin (μmol/l)	≤20	20-32	33-101	102-204	>204
Cardiovascular: Hypotension	No hypotension	MAP <70mmHg	Dopamine ≤5 ^a , dobutamine (any dose)	Dopamine >5 ^a epinephrine ≤0.1 ^a or norepinephrine ≤0.1 ^a	Dopamine >15 ^a epinephrine >0.1 ^a or norepinephrine >0.1 ^a
Haematological: Platelet count	>150	≤150	≤100	≤150	≤20
Neurological: Glasgow Coma scale score	15	13-14	10-20	6-9	<6

For SOFA score: PO₂ : FiO₂ ratio convert PaO₂ in kPa to mmHg by multiplying by 7.5

Use FiO₂ as decimal e.g. if PaO₂ = 8kPa, Convert to mmHg = 8x 7.5 = 60mmHg if FiO₂ = 0.4

60 / 0.4 = 150

^a Adrenergic agents administered for at least one hour (doses given are in μg/kg per minute). FiO₂, fractional inspired oxygen; MAP, Mean arterial pressure; PaO₂, SOFA, Sequential Organ Failure Assessment

(Doig et al., 2004).

3.7.4. Clinical outcomes

- Length of stay in ICU;
- Length of stay in hospital;
- Mortality.

3.8. Blood Sampling Procedures

3.8.1. Method

Blood sampling was done at baseline (day 0) of the study period within 12 hours from commencement of treatment with inotropes, then daily until inotrope cessation, and thereafter at day seven, following cessation of inotropic support.

For each of these assays, a maximum of 5ml of whole blood was drawn into a heparinised/EDTA vacutainer, which was chilled and kept on ice. Once drawn, blood samples were gently rocked by hand to mix the blood with anticoagulant, and then placed on ice for transport to the laboratory. All samples were centrifuged at 1000rpm for 10 minutes; and the plasma was drawn off into 1.5ml Eppendorf tubes, given a coded label, and stored at -20 to -80°C until batch analysis.

The following laboratory assays were done:

- TBARS as a marker of oxidative stress;
- Plasma vitamin C Assay (Ferric Reducing Ascorbate Assay);
- sVCAM-1 and E-selectin as markers of endothelial function.

3.9. Laboratory analysis

3.9.1. TBARS assay

3.9.1.1. Principle

This assay detects TBARS, which are products of lipid peroxidation, and are naturally present in biological specimens, such as plasma. Concentration levels of TBARS increase in response to oxidative stress (Armstrong & Browne, 1994). TBARS values are usually reported in malondaldehyde equivalents, a compound that results from the decomposition of polyunsaturated fatty acid lipid peroxides.

The TBARS assay is a well-recognised, established method for quantifying lipid peroxides; and it is useful because of its sensitivity and simplicity (Liu et al., 1997). However, compared with new gas chromatography mass spectrometric assay measuring malondialdehyde (MDA), the assay is non-specific, due to its reactivity

towards compounds other than MDA (Yagi, K., 1998). The non-specificity probably results from the acid-heating step of the assay that causes the formation of artifactual TBA/MDA-like derivatives (Jentzsch et al., 1995; Liu *et al.*, 1997).

There are also other biomarkers of *in vivo* oxidative stress and lipid peroxidation, such as isoprostanes and isofurans – with higher sensitivity and specificity (Ware et al., 2011). However, the TBARS assay is more affordable and simpler to use. It has been recommended, therefore, that the assay results be interpreted with an understanding of the limitations (Rael et al., 2004).

The practical aspect of the assay involves the following steps: The samples and blank (distilled water) are first reacted with thiobarbituric acid (TBA) at 90°C. After the 45 minute incubation, the blanks and samples are read fluorometrically at absorbance 532 and 572 nm. The TBARS concentration in nmol/ml is then calculated by subtracting the blank absorbance value from the absorbance at 532 and 572 nm, respectively, and then calculating the arithmetic mean of the two readings.

The final concentration was then calculated using the formula:

$$\text{Concentration in nmol/ml (}\mu\text{mol/L)} = \frac{([A_{532} - \text{bl}] - [A_{572} - \text{bl}] \times [500/300]) \times [1000/200]}{0.14}$$

Where:

- A_{532} and A_{572} are the absorbance values at 532 and 572 wavelengths, respectively;
- **500** is the amount of butanol used in μl per sample;
- **bl** are the values of the blanks;
- **300** is the final amount of sample read using a fluorometer;
- **1000** is the amount of microlitres equal to 1 millilitre;
- **200** is the amount of plasma in microlitres used from the original sample in the assay analysis;
- **0.14** is the conversion factor for the concentration of TBARS to nmol/ml ($\mu\text{l/L}$).

3.9.1.2. Assay reagents and method

The TBARS assay was performed, according to a modified method devised by Jentzsch et al. (1996), and then developed and optimised at the Lipidology Research Laboratory, the University of Cape Town, as follows:

Samples were thawed on ice. Distilled water was used as a blank to eliminate any background effect; and the assay was performed in duplicate.

From the plasma samples, 200µl was pipetted into an Eppendorf tube, where 25µl of butylated hydroxytoluene (BHT) (Sigma-Aldrich Corporation St. Louis, MO, United States of America), 4mM in ethanol (Merck chemicals (Pty) Limited, Gauteng, Republic of South Africa), 200µl of 2M Ortho-phosphoric acid (Sigma-Aldrich Corporation St. Louis, MO, United States of America), and 25µl of TBA (2-Thiobarbituric acid, Sigma-Aldrich Corporation St. Louis, MO, United States of America) was added.

The mixture was vortexed for 10 seconds, and then centrifuged at 13000rpm. Following the centrifugation, the mixture was heated for 45 minutes at 90°C to initiate chemical reactions. The mixture was put on ice for 2 minutes and brought to room temperature for 5 minutes. Then 500µl of n-butanol (Butan-1-ol, Merck chemicals (Pty) Limited, Gauteng, Republic of South Africa) and 50µl of saturated sodium chloride (Merck chemicals (Pty) Limited, Gauteng, Republic of South Africa) were added. The mixture was vortexed for 10 seconds and centrifuged for 2 minutes at 12000rpm. Then, 300µl of the supernatant (pink-tinged) was pipetted into a 96 well plate and read at 532 and 572 nm, using a VersaMax plate reader (Molecular Devices Corporation, California 94089, USA).

The TBARS concentration in nmol/ml was calculated, by subtracting the blank absorbance value from the absorbance at 532 and 572 nm, respectively, and calculating the arithmetic mean of the two. The final concentration was then calculated, using the formula described in the assay principle.

3.9.2. Vitamin C assay (Ferric Reducing Ascorbate Assay)

3.9.2.1. Principle

The assay is based on the principle that Fe^{3+} is reduced to Fe^{2+} by any antioxidants present. The ferrous iron is chelated with a colorimetric probe to produce a product with a strong absorbance band, which is detected between 545 and 600 nm. Adding ascorbate oxidase to the parallel samples removes any ascorbate present, leaving a background value, which is subtracted from the total value, to give the ascorbate content.

3.9.2.2. Method

Vitamin C concentration in the plasma was determined using a Ferric Reducing Ascorbate (FRASC) assay kit (Catalog #K671-100) provided by BioVision Research Products (CA 94043 USA).

A standard curve was prepared, using the standard provided with the kit; and dilutions were made using distilled water to generate 0, 2, 4, 6, 8 and 10 nmol per well of ascorbic acid.

A volume of 100 μl of each sample was then added to a paired set of wells in a 96-well plate. One of the pairs represented a well for total antioxidant present, and the other a background well (ascorbate-depleted well). 10 μl of distilled water was added to the total antioxidant well, while to the background well, 10 μl of ascorbate oxidase was added.

The plate was incubated for 15 minutes at room temperature to allow depletion of all the ascorbate. Ascorbic acid reaction mix was prepared, according to the kit protocol; and 100 μl of this mix was added to the wells containing the ascorbic acid standard and the test samples. The assay was done in duplicate; and the reading was done within 2 to 3 minutes at 593 nm using a VersaMax plate reader (Molecular Devices Corporation, California 94089, USA).

Calculations were done by first subtracting the values of the background wells from the wells with total antioxidants present. The difference was optic density, due to ascorbic acid.

Concentration (C) = Ascorbate concentration in the sample, was calculated from the following equation:

$$C = (A_t - A_b) / (\text{slope of the standard curve})/V = \text{nmol/ml} = \mu\text{M}$$

Where:

- **A_t** = absorbance of the total antioxidant well;
- **A_b** = absorbance of the background well with ascorbate oxidase;
- **Slope** = absorbance at 10 nmol standard – 0 nmol standard / 10 nmol;
- **V** = sample volume added to the reaction well (in ml).

3.9.3. Enzyme-linked immunosorbent assay (ELISA)

3.9.3.1. Principle

ELISA is a powerful method for detecting and quantifying a specific protein in a complex mixture; and it was originally described by Engval and Perlmann (1971). This method enables analysis of protein samples immobilized in a micro-plate well, using specific antibodies.

The human ELISAs are *in vitro* enzyme-linked immunosorbent assays for the quantitative measurement of specific proteins in plasma and other biological fluids. These assays employ antibodies specific to the proteins of interest. Standards and samples are pipetted into the wells; and the protein present in the samples is bound to the wells by the immobilized antibodies. The wells are then washed and biotinylated antibody is added. Washing is done again, in order to remove unbound biotinylated antibody; and then HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed and tetramethylbenzidine (TMB) is added to the wells, after which a blue colour develops. The colour change shows that the secondary antibody has bound to the primary antibody, which strongly implies an immune reaction to the test antigen. The intensity of colour change is in proportion to the amount of protein

bound. Stop solution is then added and the colour changes from blue to yellow, and the intensity of the colour is read at a specific wavelength.

3.9.3.2. Method

ELISA for soluble vascular cell adhesion molecule (sVCAM-1):

The ELISA for sVCAM-1 was performed using a commercial human sVCAM-1 ELISA kit (RayBiotech Company, GA 30092, USA).

The samples were thawed on ice; and then all the reagents were brought to room temperature. Standard solutions were made, according to protocol, to produce 60ng/ml, 20ng/ml, 6.667ng/ml, 2.222ng/ml, 0.74ng/ml, 0.247ng/ml and 0 ng/ml (animal serum with 0.09% sodium chloride). The samples were diluted 50-fold. 100µl of each standard and sample was added to the wells in duplicate. The wells were then incubated overnight at 4° C, with gentle shaking using a shaker.

The following day, the solution was discarded; and washing was done, using a wash buffer solution provided with the kit. The washing was done 7 times rather than 4 times, according to the guidelines with 5 minutes for each wash. Washing 7 times was preferred, because this was found to be the optimal number of washes that prevented sample components or antibodies cross-reacting with the blocking buffer, resulting in a high background signal. Expert opinion was sought when optimizing the assay from an experienced academic staff member from the Division of Haematology at the University of Cape Town.

After the wash, 100µl of prepared biotinylated antibody was then added to each well; and the wells were incubated for 1.5 hours to allow binding. This was done at room temperature, with gentle shaking, using a shaker.

The washing step was repeated, as previously done, after discarding the solution. 100µl of streptavidin solution was then added to each well; and the wells were incubated at room temperature, with gentle shaking for 45 minutes. The solutions were then discarded and the washing step was repeated, but this time only 4 time, according to the kit protocol, because the solutions being washed at this time had

particular specific proteins and were expected to have less unwanted proteins, if any, compared to the sample plasma.

TMB one-step substrate reagent (100µl) was added to each well; and the wells were incubated in the dark for 30 min. Then 50µl of stop solution was added; and the reading was done at 450 nm, using a VersaMax plate reader (Molecular Devices Corporation, California 94089, USA).

Calculations to determine the actual concentration of sVCAM-1 were done by plotting a curve of the standards, and using the slope to calculate the different unknown values for the samples.

ELISA for E-selectin:

The ELISA for E-selectin was performed, using the Human E-selectin ELISA kit (RayBiotech Company, GA USA).

The samples were thawed on ice; and then all the reagents were brought to room temperature. Standard solutions were then prepared, according to the kit protocol, to produce 18000pg/ml, 6000pg/ml, 2000pg/ml, 666.7pg/ml, 222.2pg/ml, 74.07pg/ml, 24.69 and blank provided with the kit (animal serum with 0.09% sodium chloride). The samples were diluted 100-fold to produce the expected concentration to be detected, and to fall within the kit's detectable range, considering the expected high levels in the sample of septic patients, as in the previous literature.

100µl of each standard and sample was added to the wells in duplicate. The wells were then incubated with gentle shaking at 4°C.

The following day, the solution was discarded; and washing was done, using a wash buffer solution provided with the kit. The washing was done 7 times, rather than 4 times, according to the guidelines, with 5-minute time intervals for each wash. Washing 7 times was preferred, because this was found to be the optimal number of washes that prevented sample components or antibodies cross-reacting with the blocking buffer, resulting in a high background signal.

After the wash, 100µl of prepared biotinylated antibody was then added to each well; and the wells were incubated for 1.5 hours to allow binding. This was done at room temperature, with gentle shaking using a shaker.

The washing step was repeated, as previously done, after discarding the solution. 100µl of streptavidin solution was then added to each well; and the wells were incubated at room temperature, with gentle shaking for 45 minutes. The solutions were then discarded; and the washing step was repeated, but this time, only 4 times, according to the kit protocol, because the solutions being washed had particular specific proteins; and they were expected to have less unwanted proteins, if any, compared to the sample plasma.

TMB one-step substrate reagent (100µl) was then added to each well; and the wells were incubated in the dark for 30 minutes. Then 50µl of stop solution was added; and the reading was done at 450 nm, using a VersaMax plate reader (Molecular Devices Corporation, California 94089, USA).

Calculations to find the equivalent concentrations of E-selectin were done by plotting a curve of the standards, and using the slope to calculate the different unknown values for the samples.

3.10. Statistical analysis

Statistical analysis of the data was done using STATA12 and STATISTICA 11 (Statsoft, USA). The Shapiro-Wilks normality test was used to test the data for normality. Summary statistics were used to provide a general description of the study population by age, sex and the source of infection. Descriptive statistics were expressed as means \pm SD and medians (IQR) for the continuous data, depending on whether the data were parametric or non-parametric. The plasma Vitamin C levels, TBARS, glucose-to-vitamin C ratio, sVCAM-1 and E-selectin levels were reported as medians (IQR) because they were non-parametric.

The Wilcoxon rank-sum test test (the Mann-Whitney test) was used to test the null hypothesis – that the two populations had equal medians in terms of investigating differences in the measured variables – including survivors and non-survivors of

septic shock, both males and females. Testing the differences between the means and the medians of the measured variables at different points in time, i.e. the baseline, day 1, and day 7, the Wilcoxon rank-sum test and the repeated measures for ANOVA or the Friedman (K-related samples) were used.

To measure the associations between the different variables; vitamin C, TBARS, sVCAM-1, E-selectin and clinical outcomes of interest, the Spearman's test of association was used. A p-value of less than 0.05 was considered to be statistically significant.

CHAPTER 4: RESULTS

4.1. Participants

Recruitment for the study was done in the period between March 2012 and December 2012. The recruitment of the participants was done by one of the designated research team members on call, on a rotational basis – actively searching for potential participants. Referral of potential participants was also done by the ICU staff. During the study period, a total of 80 patients were screened for possible inclusion in the study. Of these, 25 patients could be included. Table 4.1 summarises the participant's demographic data and their baseline characteristics.

Table 4.1: Participant demographics (n = 25)

Source of Sepsis	Intra-abdominal	8/25 (32%)
	Respiratory	7/25 (28%)
	Necrotizing Fasciitis	4/25 (16%)
	Polytrauma with secondary infection	3/25 (12%)
	Soft Tissue	3/25 (12%)
Gender (males / females)	7/18 (28% males, 72% females)	
Mean age (years)	49 ± 15.6	
Mean BMI (kg/m ²)	28.2 ± 6.4	
Mean APACHE II Score	20.1 ± 10.5	
Mean baseline SOFA Score	10.6 ± 2.8	
Mean lowest Mean Arterial Pressure (mmHg)	54.9 ± 10.9	
Survived / Died	16 (64%) / 9 (36%)	
Median length of ICU stay (days)	6 (4-10)	

APACHE II: Acute Physiology and Chronic Health Evaluation

SOFA Score: Sequential Organ Failure Assessment Score

BMI: Body Mass Index

Those study participants who had surgical diagnoses represented 80% (n=20) of the study group; while 16% (n=4) had internal medical diagnoses; and 4% (n=1) had a gynaecological diagnosis. The mean APACHE score of the participants was 20.1 ± 10.5, with no statistical difference between surgical 17.9 ± 11.1 and medical patients 24.125 ± 10.6. The baseline SOFA is consistent with patients suffering septic shock – given that most of these patients had multi-organ dysfunction. Comparing the SOFA

score between the survivors and the non-survivors, it was found that the baseline SOFA score was lower in survivors 9.75 ± 2.7 compared to non-survivors of septic shock 12.2 ± 2.1 ; and this difference was statistically significant, using a two-sample t-test ($p=0.014$). This indicates that the non-survivors were more severely ill than the survivors at baseline.

The mortality rate in the patients of this study was 36% and this is comparable to the mortality rate associated with septic shock worldwide of 20%-51% (Kethireddy & Kumar, 2012; Labelle et al., 2012). The median length of ICU stay was six days (4-10) with no significant difference between the survivors and the non-survivors of septic shock ($p = 0.6$).

4.2. Screen failures

From the 80 patients who were screened, 55 patients were excluded from the study. Figure 4.1 indicates the reasons for their exclusion. Most of the patients were not included into the study because they were not timeously referred. However, a considerable proportion was also excluded, because they were either on dialysis, or receiving parenteral nutrition.

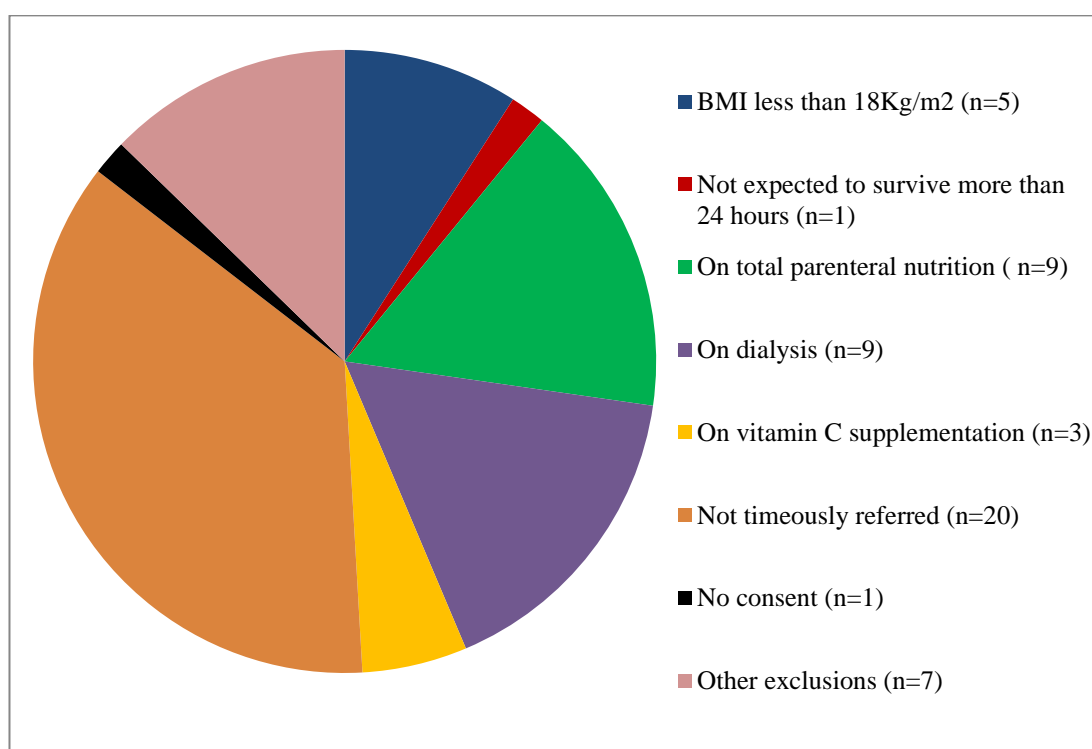
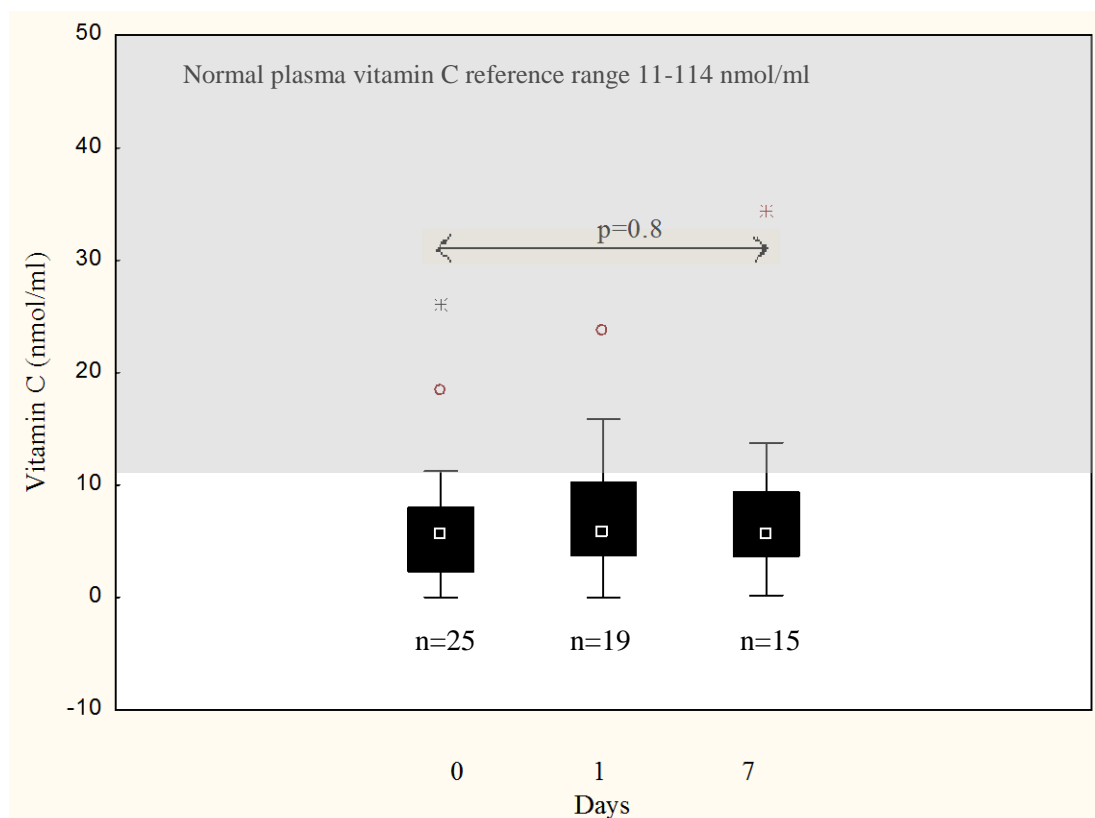


Figure 4.1: Screen failures

4.3. Plasma vitamin C levels

The first objective of the study was to measure the plasma vitamin C status in the patient group. Plasma samples drawn at baseline, on day one and on day seven, and were analysed for vitamin C, using the ferric reducing ascorbate assay. Figure 4.2 shows the box plots of the plasma-vitamin C levels at the different times of the study period.

For the duration of the study, the median (IQR) vitamin C levels were low in the participants with no significant change [baseline (5.65 nmol/ml [2.31 – 8.02]), on day one 5.9 nmol/ml (3.73 – 14.15), and on day seven 5.61 nmol/ml (3.69 – 9.35) ($p = 0.83$)]. One patient at baseline and another at day one of the study period had undetectable levels of vitamin C in their plasma. Only three of the participants had vitamin C levels within the normal reference range of 11 – 114 nmol/ml at baseline. At day one, five patients; and at day seven, three patients had levels within the normal reference range.



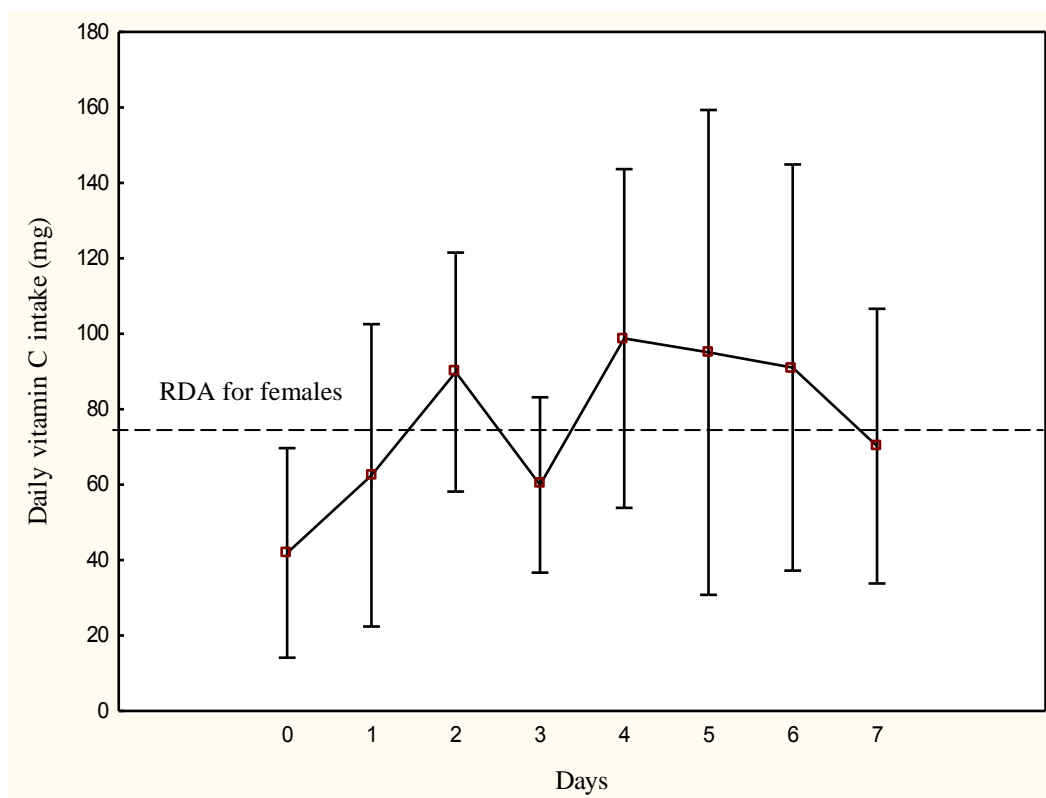
Grey band represents the normal reference range (11-114nmol/ml)

°= outliers, * = extremes

Figure 4.2: Vitamin C levels over the study period

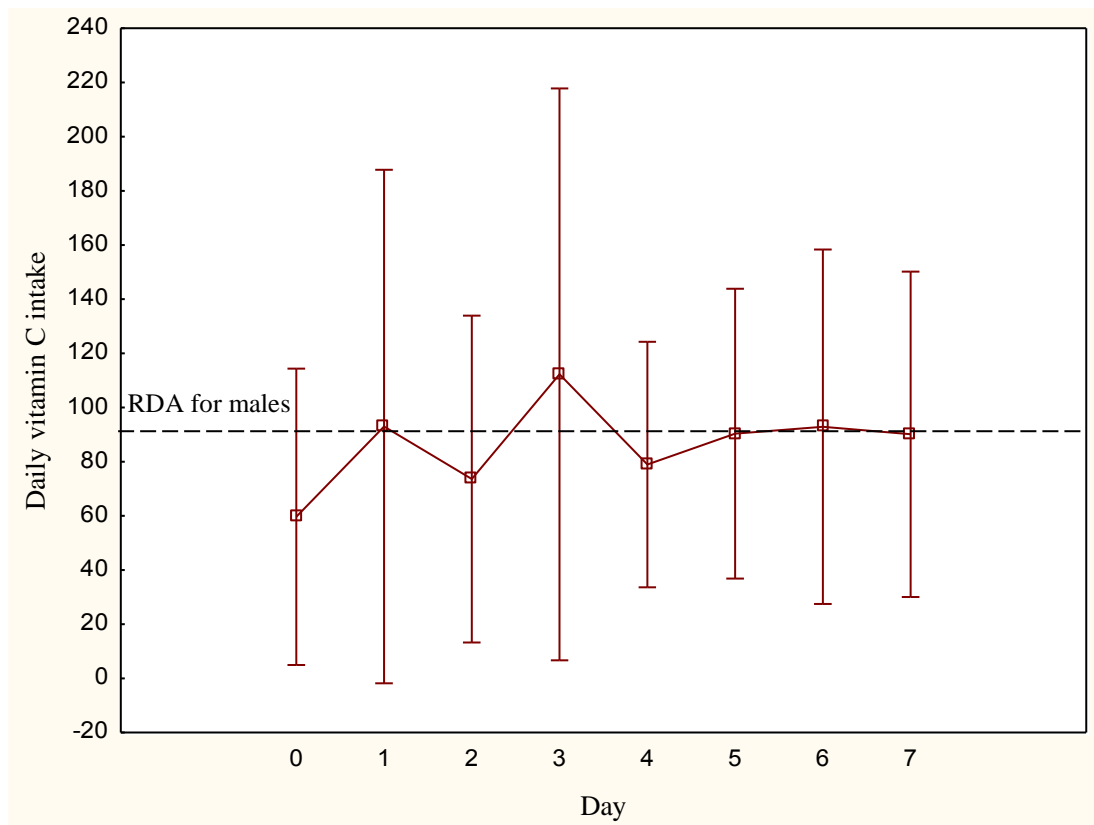
The Wilcoxon rank-sum test was used to test the difference in the vitamin C levels between the males and the females. There was no significant difference in the vitamin C plasma levels between males and females at any of the time points ($p = 0.8$ at baseline; $p = 0.3$ at day one; and $p = 0.3$ at day seven), respectively. Similarly, no significant differences were found in the baseline plasma vitamin C concentrations in survivors and non-survivors of septic shock ($p = 0.9$).

Figure 4.3 depicts the trend of vitamin C daily intake over the study period in A (females) and B (males). Of note is that the mean vitamin C intake from all the sources in female patients fell below the recommended daily allowance (RDA), within the first 48 hours (75mg/day) following diagnosis of septic shock; and the readings were only above the RDA on days: two, four, five and six. Males had oscillating vitamin C intake, and had intake levels below the RDA on all the study days except days one, three and six.



Recommended daily allowance (RDA) for healthy females 75mg/day

A. (Females)



Recommended daily allowance (RDA) for healthy males: 90mg/day

B. (Males)

Figure 4.3. Daily mean vitamin C intake (mg) in ICU over the study period in A (females) and B (males)

4.4. Plasma TBARS as a marker of oxidative stress

The second objective of the study was to measure the plasma concentration of TBARS as a marker of oxidative stress. TBARS assay was analysed at the same time points of the study as the vitamin C above (baseline, day one and day seven).

Table 4.2 shows the median TBARS levels at the indicated time periods of the study. At baseline, the median TBARS levels were more than four times higher than the upper reference range values. The TBARS levels continued to be high at day one, with no statistically significant reduction at day seven, when tested with the Friedman ANOVA.

Table 4.2: Median (IQR) plasma TBARS (nmol/ml) over the study period

	Study day			p- value (Friedman ANOVA)
	Baseline (n=25)	Day 1 (n=19)	Day 7 (n=15)	
Median (IQR) TBARS (nmol/ml)	19.5 (14-37)	20.4 (13-64)	13.3 (9-18)	0.52

Reference range 1.86-3.91 nmol/ml (Miller et al., 2012)

However, as depicted in Figure 4.4, non-survivors of septic shock had higher median TBARS levels at baseline (43.8 nmol/ml [23.6 – 47.7]) than did the survivors (16.9 nmol/ml [11.9 – 21.7]), ($p = 0.008$).

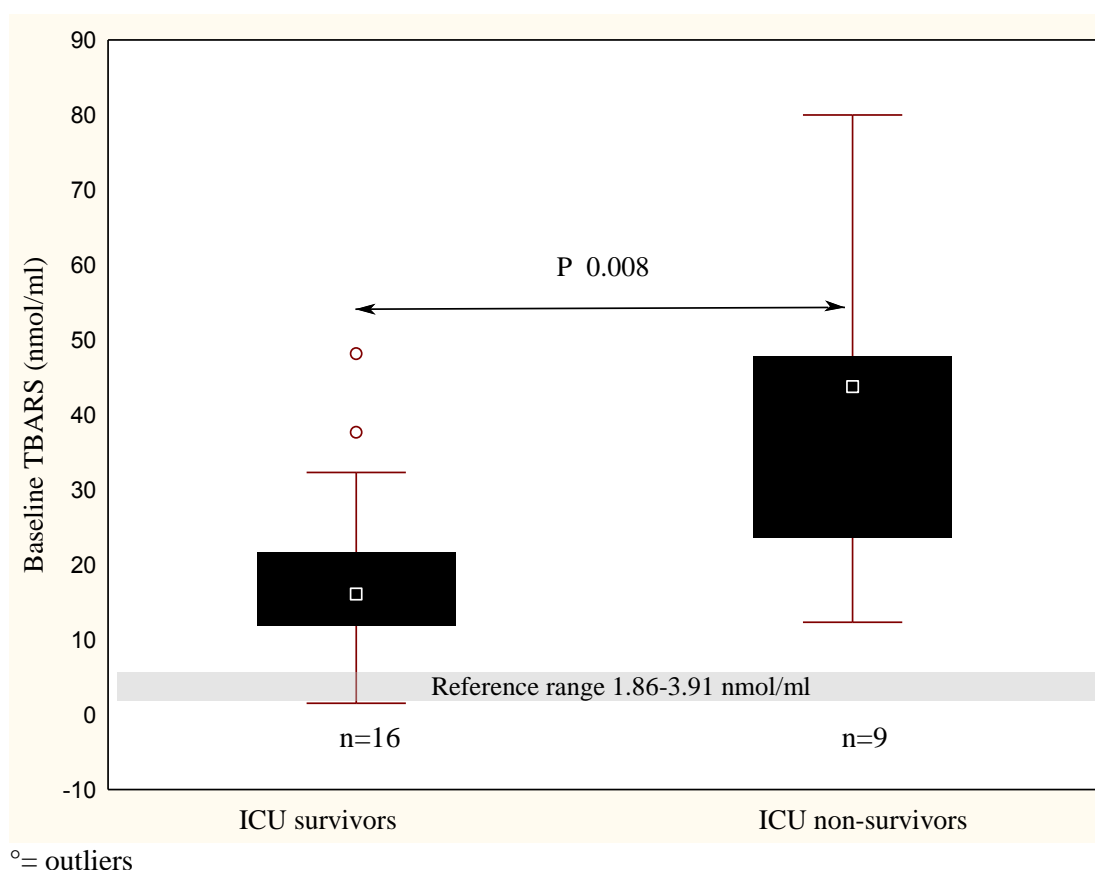


Figure 4.4. Oxidative stress levels (measured as TBARS) in survivors and non-survivors of ICU

This indicates that the non-survivors of septic shock had more than double the levels of oxidative stress than did the survivors. Despite this, even the survivors still had a

median TBARS concentration of approximately four times higher than the upper limit of the reference range.

4.5. Plasma glucose

Daily blood glucose measurements were done, as part of the routine care in ICU. At baseline, day one and day seven, blood glucose measurements were done specially for the calculation of blood glucose-to-vitamin C ratio – for the study purposes.

Table 4.3 shows the median (IQR) daily highest, lowest and mean blood glucose records for the designated days. The table shows that the mean blood glucose was well-controlled in the ICU over the study period, when compared to the Groote Schuur ICU protocol target range (5 – 8 mmol/l). The mean blood glucose was above the ICU target range at baseline, but significantly reduced to the ICU target values at day one and day seven, respectively. There was, however, no statistically significant change in either the median highest blood glucose ($p = 0.2$), or the median lowest blood glucose ($p = 0.8$) level over the study period. No difference was shown in the baseline mean blood glucose between survivors 9.4 mmol/l (7.5 – 10.5) and non-survivors 7.7mmol/l (6.5 – 8.8) ($p = 0.22$).

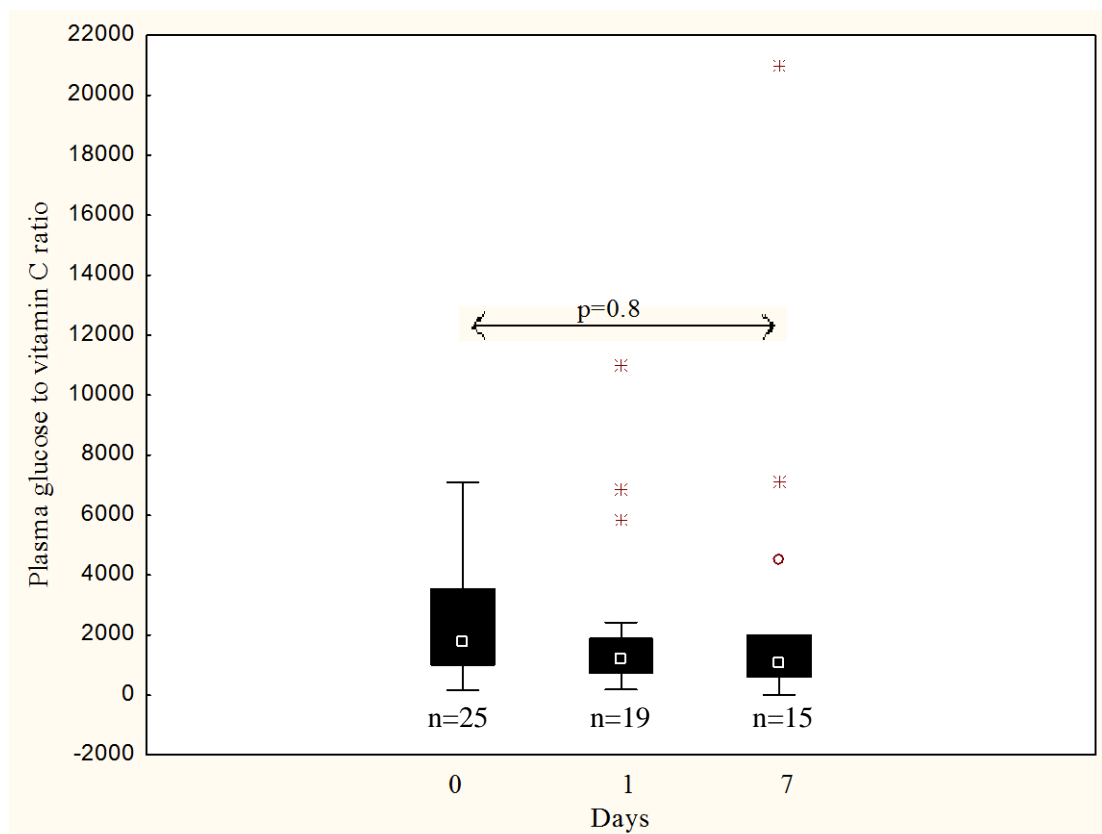
Table 4.3: Median (IQR) blood glucose measurements (mmol/l) over the study period

Type of Blood glucose	Study day			p-value (ANOVA) change over study period
	Baseline	Day 1	Day 7	
Highest reading	12 (9.3-14.3)	10.3 (8.45-11.7)	8 (7.25-10)	0.2
Lowest reading	5.5 (4.6-6.4)	5.25 (3.85-6.25)	5.75 (4.7-6.1)	0.8
Mean reading	8.84 (7.3-10.1)	7.78 (5.95-8.55)	7.19 (6.4-7.8)	0.03

Groote Schuur Hospital ICU blood glucose target range (5-8 mmol/l)

4.6. Plasma glucose to vitamin C ratio

The subsequent step was to determine the glucose-to-vitamin C ratio in the study group. Figure 4.5 depicts the median (IQR) of the ratios over the study days. It should be noted that there was a high median (IQR) ratio of glucose-to-vitamin C at baseline (1756 (998 – 3539)), with no significant change at the other time points (day one [1178 (727 – 1877)] and day seven (1043 [593 – 2000])).



Normal plasma glucose to vitamin C expected ratio = 88 (Price and Reynolds, 2001)

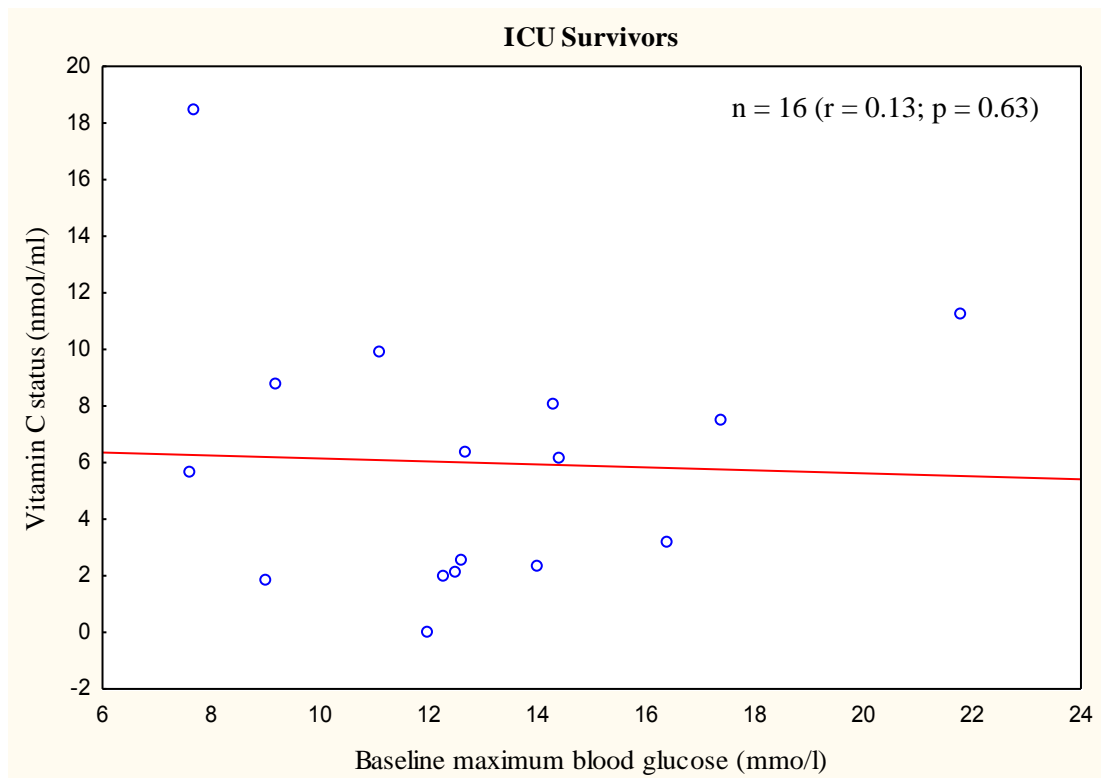
°= outliers, * = extremes

Figure 4.5: Glucose to vitamin C ratio over the study period

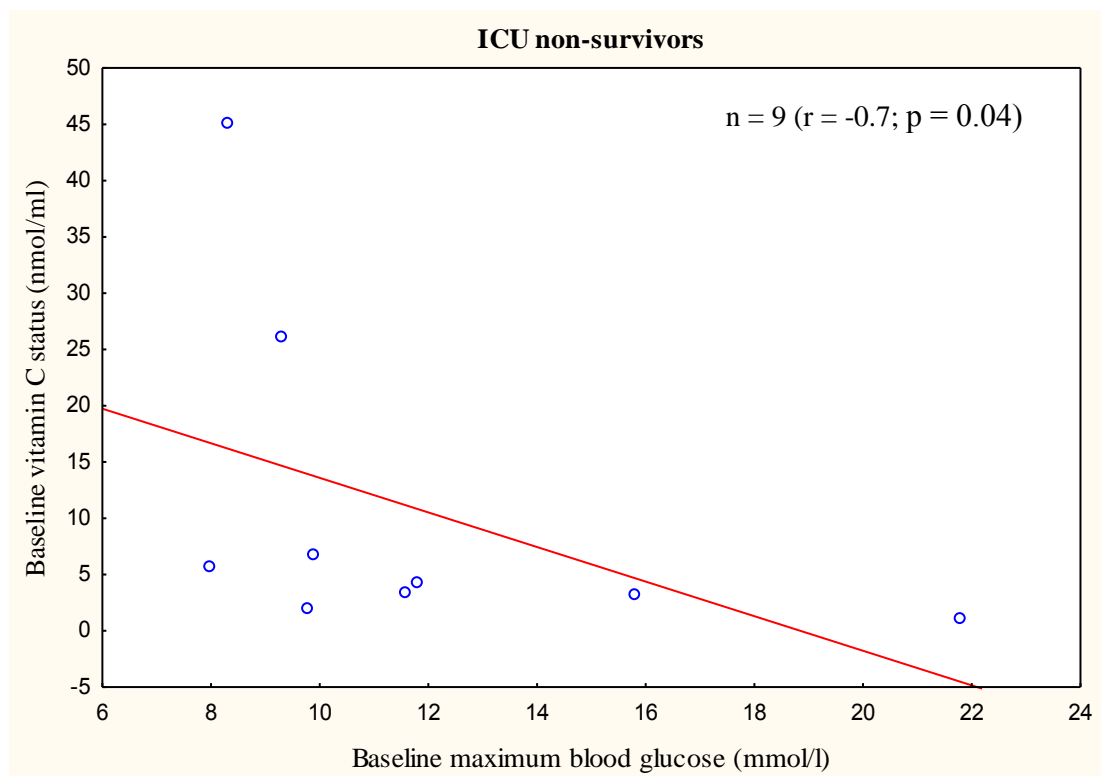
4.7. Association between plasma glucose and vitamin C

The next objective of the study was to investigate the relationship between plasma glucose and vitamin C. There was no correlation between glucose and vitamin C status at any of the time points [baseline ($r = -0.06$, $p = 0.8$), day one ($r = -0.2$, $p = 0.5$), and day seven ($r = 0.06$, $p = 0.9$)]. Further analysis, as illustrated in Figure 4.6, showed that there was no significant correlation between the baseline vitamin C and the maximum blood glucose in the survivors (Fig 4.6A), ($r = 0.13$, $p = 0.63$).

In the non-survivors, however (Fig 4.6B), there was a significant negative correlation ($r = -0.7$, $p = 0.04$).



A. (ICU survivors)

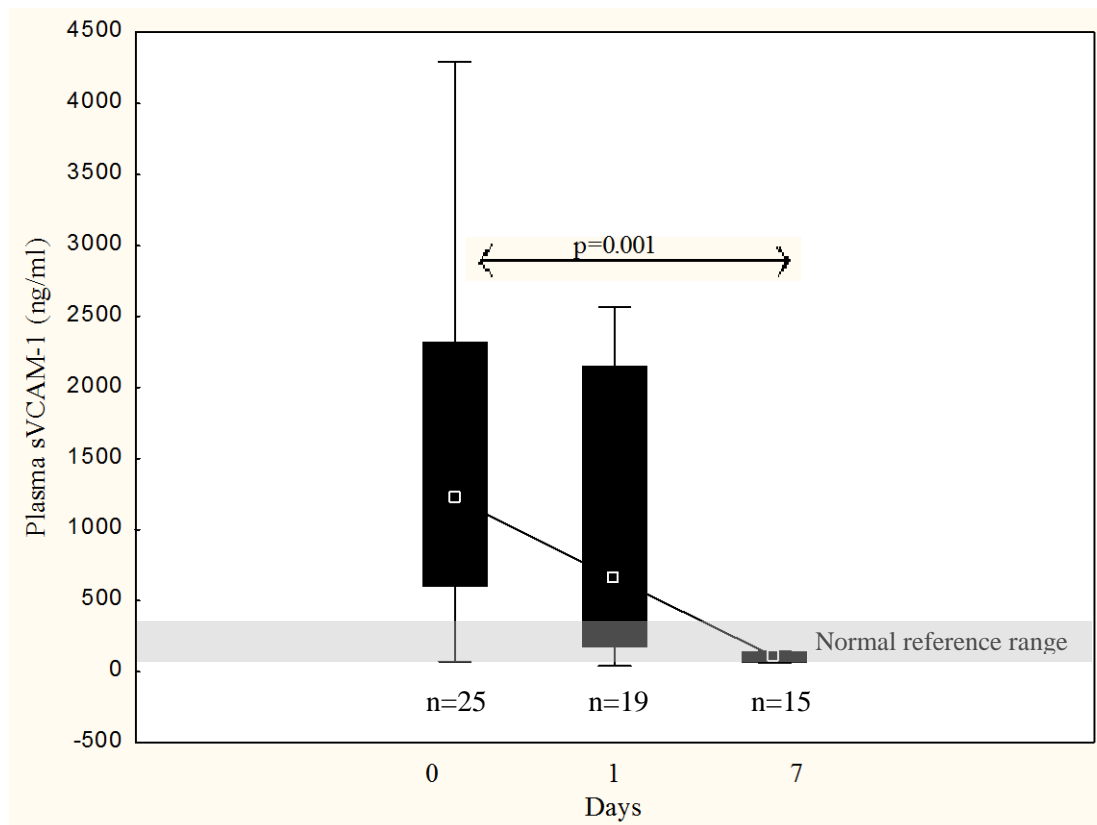


B. (ICU non-survivors)

Figure 4.6. Correlation between baseline vitamin C status and maximum blood glucose in A (ICU survivors) and B (ICU non-survivors)

4.8. Biomarkers of endothelial function

The fourth objective of the study was to measure the circulating markers of endothelial function. Plasma soluble VCAM-1 and E-selectin were measured using commercial ELISA kits.

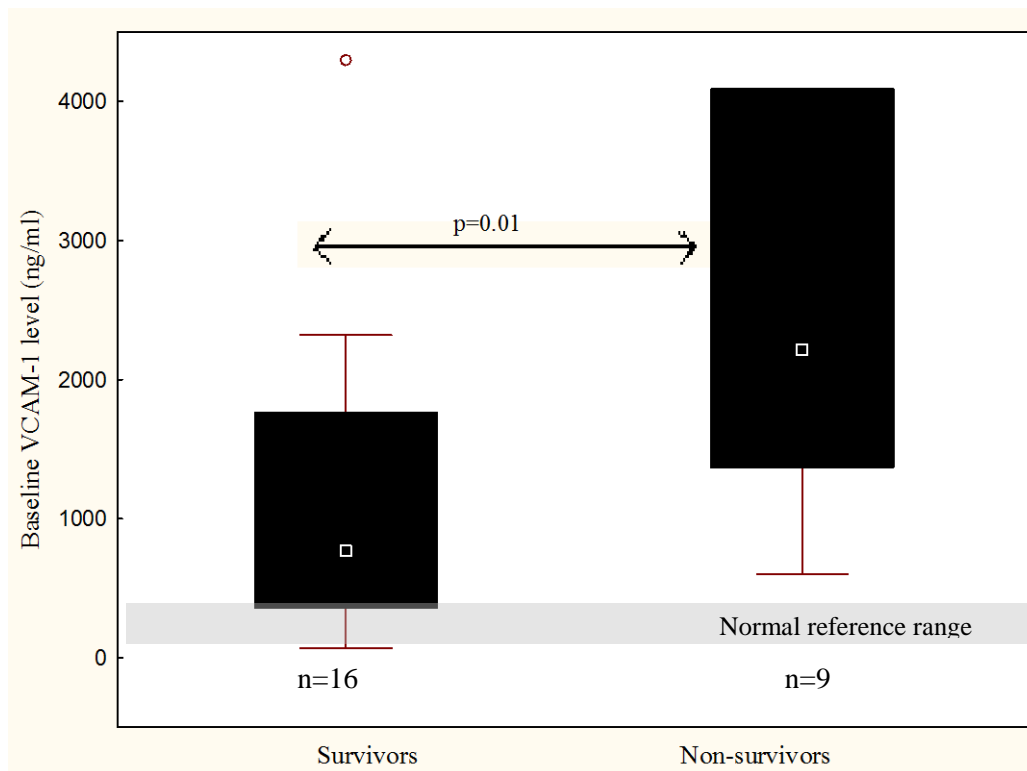


Normal reference range 72 – 349 ng/ml (Whalen et al., 2000)

Figure 4.7. Plasma sVCAM-1 concentrations over the study period

As illustrated in Figure 4.7, the median (IQR) plasma sVCAM-1 levels were raised at baseline (1219 ng/ml [603 – 2319]) but decreased significantly at day one (657 ng/ml [177 – 2149]) and day seven (105 ng/ml [67 – 135]) when tested with Friedman's ANOVA ($p = 0.001$) against day 0).

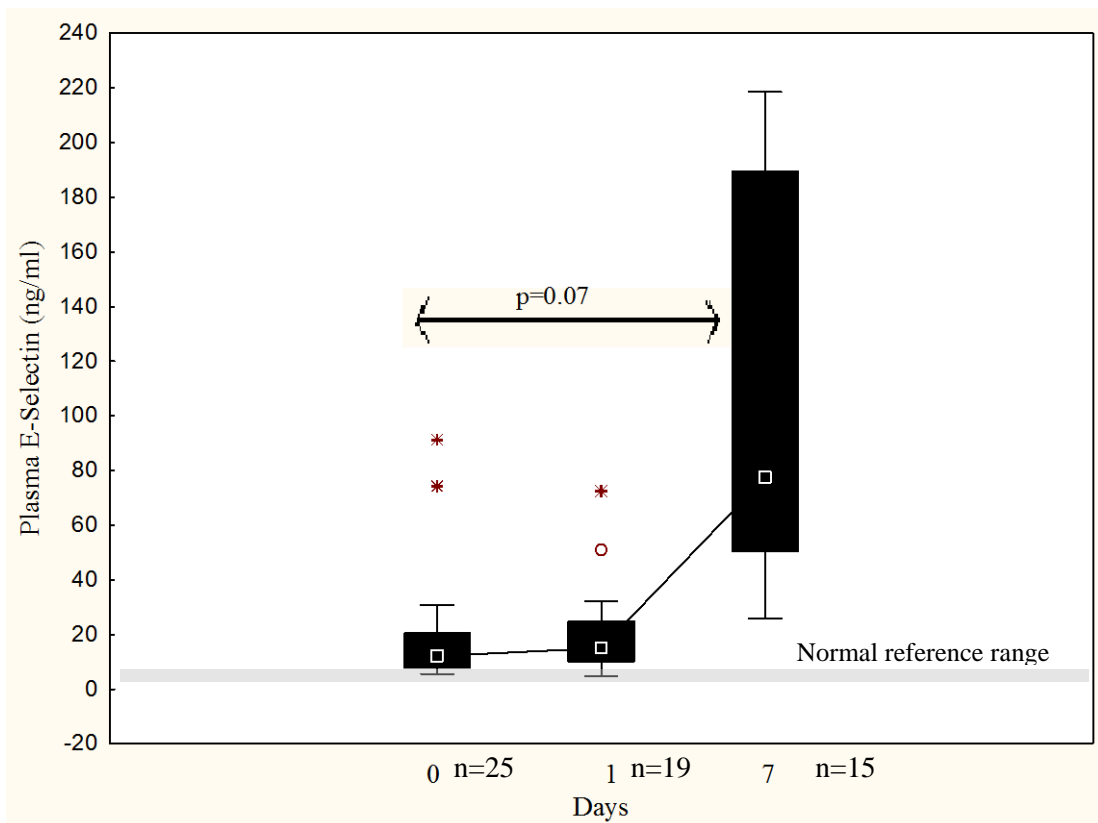
Figure 4.8 compares the baseline sVCAM-1 levels between the survivors and the non-survivors. It was noted that there was a significant difference in the baseline sVCAM-1 levels, which were higher in non-survivors (2212 ng/ml [1370 – 4081]) than in the survivors of septic shock (767 ng/ml [357 – 1764]), ($p = 0.01$).



Normal reference range 72 – 349 ng/ml (Whalen et al., 2000)

Figure 4.8. Baseline sVCAM-1 levels between survivors and non-survivors

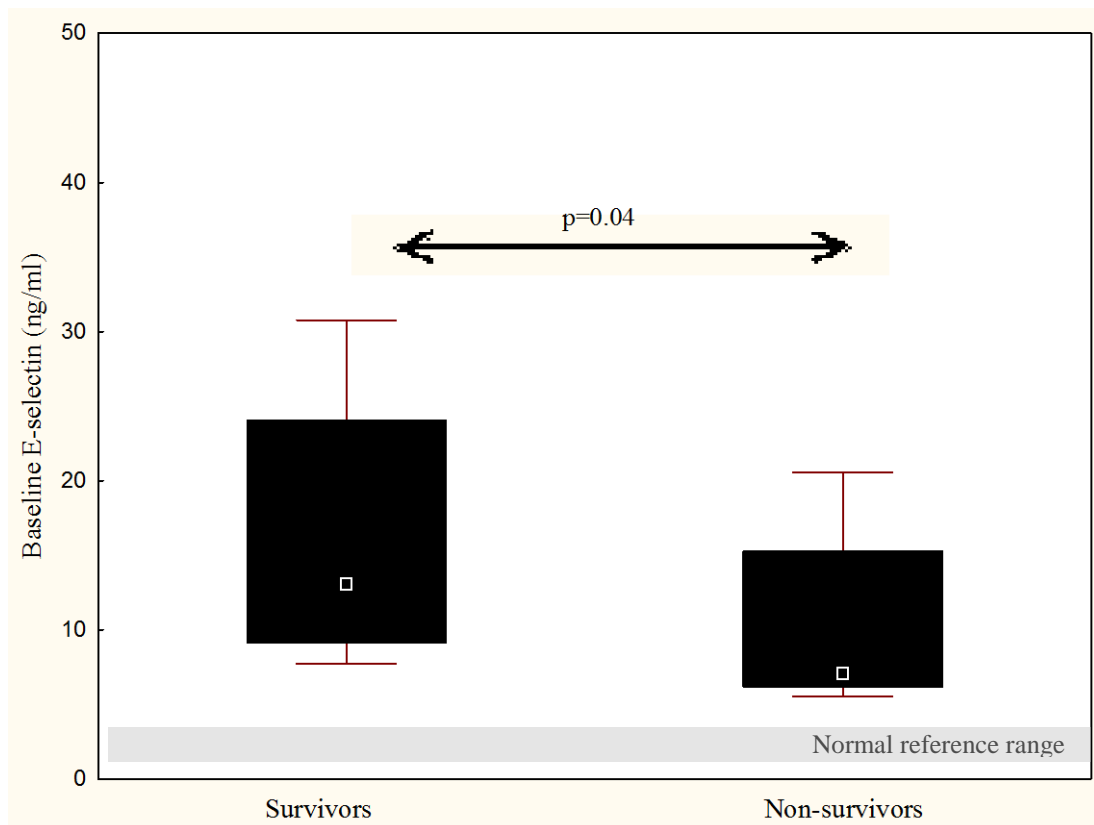
Figure 4.9 shows the plasma E-selectin levels in the patients. There was a significant increase in the median levels from day one to day seven ($p = 0.003$). Figure 4.10 displays a comparison of E-selectin levels between the survivors and the non-survivors at baseline. The E-selectin levels were significantly higher in survivors 13.1ng/ml (7.8 – 24.1) than they were in non-survivors 7.1ng/ml (6.2 – 15.3) at baseline ($p = 0.04$).



Normal reference range for E-selectin 0.93 – 2.8ng/ml (Newman et al., 1993)

°= outliers, * = extremes

Figure 4.9: Plasma E-selectin concentrations over the study days



Normal reference range for E-selectin 0.93 – 2.8ng/ml (Newman et al., 1993)

Figure 4.10. Comparison of plasma E-selectin levels between survivors and non-survivors at baseline

There was no correlation between the plasma sVCAM-1 and E-selectin at any of the time points of the study; baseline ($r = -0.3$, $p = 0.15$), day 1 ($r = 0.07$, $p=0.8$), and day 7 ($r = 0.7$, $p = 0.09$).

4.9. Correlation between vitamin C and TBARS

The other objective of the study was to investigate the relationship between vitamin C and TBARS, as a marker of oxidative stress. There was no significant correlation between vitamin C and TBARS levels at baseline ($r = 0.15$, $p = 0.47$) or day one ($r = 0.16$, $p = 0.51$). At day seven, however, there was a negative correlation approaching statistical significance ($r = -0.507$, $p = 0.06$).

4.10. Association between endothelial function markers and TBARS as a marker of oxidative stress

We also investigated the correlation between E-selectin and sVCAM-1, respectively, as markers of endothelial dysfunction, and TBARS as a marker of oxidative stress.

Table 4.4: Association between endothelial function markers and TBARS as a marker of oxidative stress

Study day	sVCAM-1 vs. TBARS		E-selectin vs. TBARS	
	Spearman's rho	p-value	Spearman's rho	p-value
Baseline	0.45	0.02	0.08	0.7
Day 1	0.57	0.01	0.16	0.5
Day 7	0.6	0.01	-0.08	0.78

As portrayed in Table 4.4 above, there was a significant positive correlation between sVCAM-1 and TBARS at all the time points of the study – thereby indicating that increased sVCAM-1 levels were associated with higher levels of oxidative stress. There was, however, no significant correlation observed between E-selectin and TBARS at any of the time points; and the associations were very weak.

4.11. Association between endothelial function markers and vitamin C status

We also investigated the correlation between sVCAM-1, as well E-selectin and vitamin C status. No correlation was found between sVCAM-1 and vitamin C status at baseline ($r = -0.02$, $p = 0.09$), day one ($r = -0.18$, $p = 0.4$), or day seven ($r = 0.16$, $p = 0.58$). Similar results were found when the correlation between E-selectin and vitamin C was tested at baseline ($r = 0.08$, $p = 0.69$), day one ($r = 0.16$, $p = 0.7$), and at day seven ($r = -0.08$, $p = 0.78$).

4.12. Association between biomarkers and clinical markers

4.12.1. Association between baseline TBARS, sVCAM-1 and SOFA Score

Figure 4.11 shows the association between baseline TBARS (left y-axis), sVCAM-1 (right y-axis) and Sequential Organ Failure Assessment score (x-axis). Increased TBARS levels were associated with high SOFA scores ($r = 0.47$, $p = 0.02$). Interestingly, baseline high sVCAM-1 levels were also associated with high SOFA scores ($p = 0.02$, $r = 0.45$).

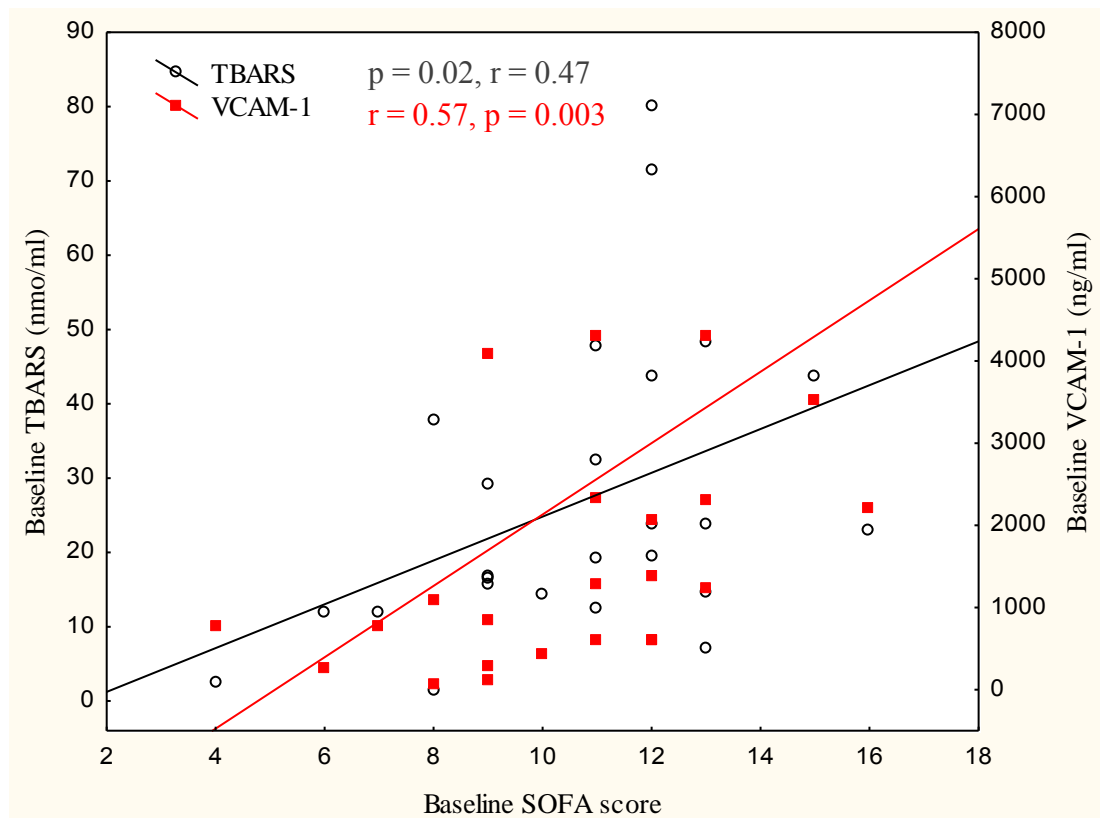


Figure 4.11: Correlation between baseline TBARS and SOFA score, baseline sVCAM-1 and SOFA score

4.12.2. Association between baseline TBARS and intravenous fluid requirement and Inotrope-free days

Figure 4.12 portrays the association between baseline TBARS (x-axis), as a marker of oxidative stress and inotrope-free days at day seven (left y-axis), and the intravenous fluid requirements (right y-axis). There was a negative association between Inotrope-free days at day seven and TBARS ($r = -0.44$, $p < 0.015$) in the total group. Baseline IV fluid volume was positively associated with TBARS in non-survivors ($r = 0.75$, $p = 0.02$).

This illustrates that patients with increased oxidative stress required more days on inotropic support, and had increased baseline intravenous fluid requirements.

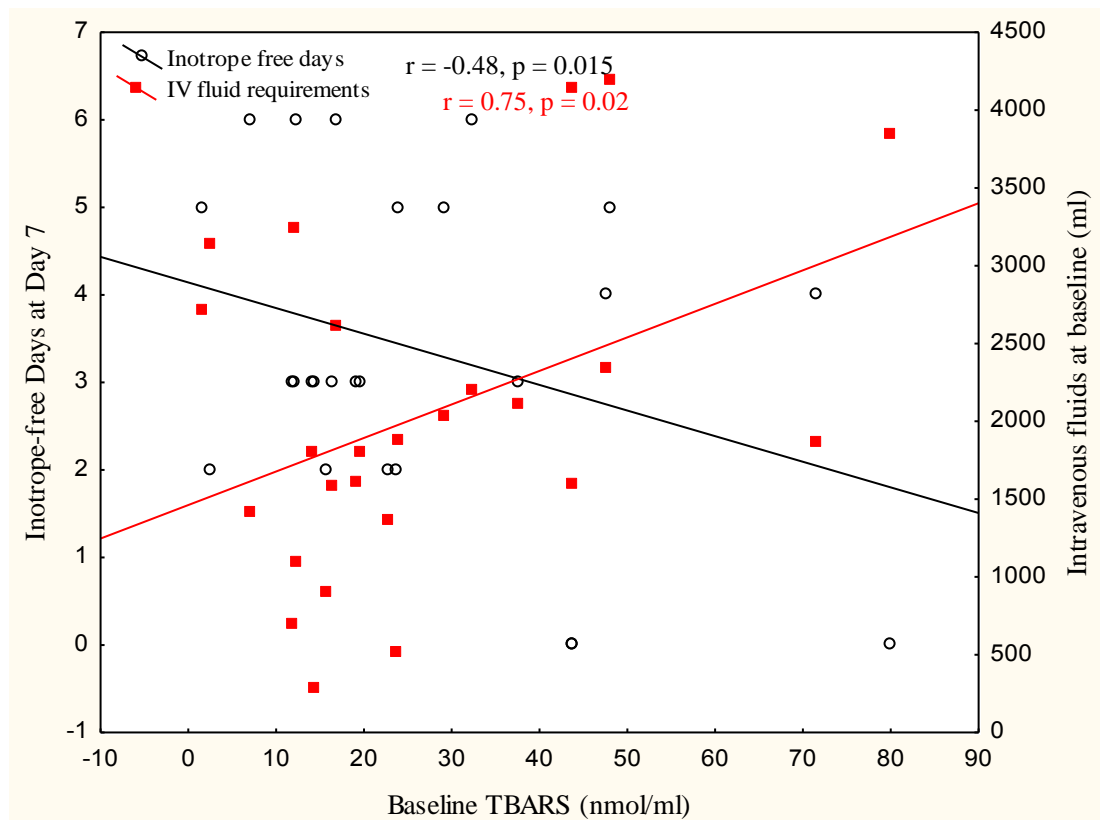


Figure 4.12: Correlation between baseline TBARS and Inotrope-free days at day 7, and baseline TBARS and baseline intravenous fluid requirements

4.13. Summary of results

This study has shown that septic shock is associated with low levels of plasma vitamin C, which persisted until day seven after the cessation of inotropic support. There was also evidence of oxidative stress in the patients, marked by increased levels of TBARS; and there was no significant reduction in these levels at day seven of the study. Non-survivors in this study had increased levels of oxidative stress and organ failure compared to the survivors.

The results of this study have also shown that the plasma glucose-to-vitamin C ratios were higher than the normal expected ratio at all of the study days. As regards plasma biomarkers of endothelial dysfunction, both markers (sVCAM-1 and E-selectin) were high at baseline. However, sVCAM-1 levels were significantly higher in non-survivors than in survivors; but this difference was not observed in the E-selectin levels. Furthermore, the sVCAM-1 levels fell significantly at day one, and normalized at day seven; while the E-selectin levels were constantly raised at day one, as at baseline, and further increased significantly at day seven.

No association was found between the biomarkers of endothelial dysfunction and those of vitamin C levels. Both sVCAM-1 and TBARS associated positively to SOFA score; and increased TBARS levels were associated with increased requirements for intravenous fluids for resuscitation, and an increased number of days on inotropes.

Table 4.5 summarises the major differences in the study variables between survivors and non-survivors.

Table 4.5: Summary table of baseline characteristic differences between survivors and non-survivors

	Variables					
	SOFA score	Vitamin C status (nmol/ml)	TBARS (nmol/ml)	sVCAM-1 (ng/ml)	E-selectin (ng/ml)	Correlation (vitamin C vs maximum glucose)
Survivors	9.8 ± 2.7	5.9 (2.2-8.4)	16.9 (11.9-21.7)	767 (357 – 1764)	13.1 (7.8 – 24.1)	r = 0.13 p = 0.63
Non-survivors	12.2 ± 2.1	4.3 (3.2-6.7)	43.8 (23.6-47.7)	2212 (1370 – 4081)	7.1 (6.2 – 15.3)	r = -0.7 p = 0.04
p value where applicable	0.014	0.9	0.008	p = 0.01	0.04	

CHAPTER 5: DISCUSSION

This is the first study in a South African setting to investigate vitamin C status, oxidative stress levels, hyperglycaemia, and their association with endothelial dysfunction in patients with septic shock. In summary, the results present a picture of critically ill patients with a profound vitamin C deficit in the context of extreme oxidative stress – both of which persisted after the resolution of the acute period of septic shock. Furthermore, the findings indicate that these responses differ between survivors and non-survivors. The non-survivors appeared to have a more exaggerated response.

Increased oxidative stress and endothelial damage in the patients were associated with increased organ dysfunction, as marked by the SOFA score. We also demonstrated higher plasma glucose-to-vitamin C ratio in these patients, indicating hyperglycaemia, low vitamin C, or the occurrence of both. It was found that oxidative stress correlated with the biomarkers of endothelial damage, and with clinically relevant indicators of the extent of shock, i.e. the duration of inotropic support and intravenous fluid requirement.

These results will be discussed further in the remainder of this chapter, and the relevance for the clinical care of patients with septic shock will be suggested.

5.1. Study participants

The sample population in this study was representative of a well-defined group of critically ill patients with septic shock, and acutely deranged physiology. At baseline, prior to study entry, all the study participants met the SIRS criteria and the specified criteria for septic shock, the definition of which is persistent sepsis-induced hypotension – despite adequate fluid resuscitation (Dellinger et al., 2013).

The lowest mean arterial blood pressure for these patients was 54.9 ± 10.9 mmHg and they all required inotropic support due to septic shock at baseline. The baseline SOFA score was 10.6 ± 2.8 . This SOFA score is consistent with the expected degree of multi-organ dysfunction and/or failure known to occur with septic shock (Grønlykke, Brandstrup & Perner, 2012). The Acute Physiology and Chronic Health

Evaluation (APACHE) II score was 20.1 ± 10.5 , indicating that the patients were systemically stressed.

The mortality rate for septic shock in our study of 36% is within the global range of mortality of 20%-51% for this diagnosis (Kethireddy & Kumar, 2012; Labelle et al., 2012). The non-survivors had a significantly higher baseline mean SOFA score than did the survivors, which is consistent with the known prognostic significance of the SOFA score (Bale et al., 2013; Jones, Trzeciak & Kline, 2009).

5.2. Vitamin C status

The vast majority (88%) of these patients had low baseline vitamin C levels. The median vitamin C levels were 5.65nmol/ml, which is only 51% of the lower limit of reference range (11nmol/l), below which manifestations of clinical vitamin C deficiency syndromes could be expected if the status remained at this level chronically.

The cause of these very low plasma vitamin C levels in our study could be as a result of three major factors. The first of these factors is critical illness. It has been established that critical illness is associated with oxidative stress, in which there is increased utilisation of antioxidant micronutrients, including vitamin C (Doise et al., 2008; Heyland et al., 2013; Long et al., 2003). The oxidative stress, and hence the antioxidant utilisation, is further increased in patients with SIRS, such as in septic shock (Alonso de Vega et al., 2002). The patients from the current study were critically ill, with a severe form of SIRS-exhibiting condition and increased markers of oxidative stress. It could be expected, therefore, that there would be an accelerated oxidation of ascorbate at a rate that exceeds reduction – due to increased oxidative stress (Galley, Davies & Webster, 1996; Galley et al., 1997; Spada et al., 2008). Damaged proteins, due to trauma or sepsis-associated oxidative stress, and extracellular ferritin, may be sources of redox-reactive iron that also oxidizes ascorbate in blood and interstitial fluid (Deubzer et al., 2010; Du, Cullen & Buettner, 2012).

Vitamin C losses from plasma are also due to the redistribution of blood volume from the intravascular to the extravascular space – with the loss of vitamin C

therewith (Wilson, 2013). Losses can also occur through dialysis, since vitamin C is water soluble (Coveney et al., 2011; Raimann et al., 2013), but this is not applicable to this study, since the patients on dialysis were excluded to remove this confounding factor.

Thirdly, very low baseline vitamin C levels in this study could be due to low plasma vitamin C prior to the onset of septic shock. There is a lack of recent South African data on the vitamin C status in healthy people. Segal et al. (1995) demonstrated that there was a significant difference in the median vitamin C levels between healthy controls from Manchester, United Kingdom (74 nmol/ml) and Johannesburg, South Africa (14 nmol/ml) ($p < 0.001$), respectively. Low dietary intake of micronutrients, including vitamin C, has been reported in most parts of South Africa (Linster & Van Schaftingen, 2007; MacIntyre et al., 2002). At least some of the South African population may, therefore, be predisposed to low vitamin C levels in acute illness – due to a pre-existing nutritional compromise in the diet. Septic shock then further worsens the already suboptimal plasma vitamin C levels – due to the increased demands associated with the disease condition (Berger, 2006).

In addition to the above factors, low vitamin C levels were persistent, possibly due to inability to restore the normal plasma levels nutritionally while in ICU. The nutrition (from all sources) delivered to the patients of this study had a mean upper range of 120 mg of vitamin C at all the time points of the study, and did not replenish the vitamin C plasma levels. However, it should be kept in mind that patients who were on TPN during screening were not included in the study.

In a review of vitamin C requirements in critically ill patients, Berger (2009) commented that only parenteral vitamin C administration of not less than 3g/day for three days would be adequate to bring the plasma vitamin C up to normal. This recommendation was based on the data from acute surgical patients.

The vitamin C levels in our study did not resolve at day seven, following the episode of septic shock. This finding confirms another published study, where the vitamin C levels in patients with severe sepsis were only restored to normal range (11 – 114 nmol/ml) between day seven and three months after the episode (Andresen et al.,

2008). These results mean that the low vitamin C status associated with severe sepsis persists for at least a week, unless a targeted intervention towards normalising the levels is offered.

These results have some clinical relevance. The question lies in the extent to which this acute, very low vitamin C status could have on clinical outcome. It is known that vitamin C is a potent water-soluble antioxidant, and that it is involved in normal vascular function (Halliwell, 1997b; Wilson, 2009). It is difficult, given our study design, to ascertain exactly what the influence of vitamin C levels as low as we have demonstrated, might be on the clinical outcome of the patients.

It has been demonstrated in previous animal and human studies that supplementation with high doses of vitamin C, and replenishing the levels in critical illness and septic shock improved the outcome (Gaut et al., 2006; Tanaka et al., 2000; Tymł, Li & Wilson, 2008). It is plausible that maintaining the vitamin C levels at normal and even above, is an important factor that has the potential to improve the patients' outcome. Considering this, the persistence of deficient levels of vitamin C in our study population is of concern – but it is not unexpected – as supplementation with high doses of vitamin C was not included in the current study.

5.3. The extent and effects of oxidative stress in septic shock

Since vitamin C plays a central role as an antioxidant, it was important to measure the oxidative stress in the patients. The results of this study showed that there was increased oxidative stress at baseline in the study participants, as marked by increased plasma TBARS 19.5nmol/ml (14 – 37) (reference range = 1.86 – 3.91nmol/ml).

The oxidative stress was evident in all the study participants. Literature has previously shown that critical illness, regardless of cause, is associated with oxidative stress (MacDonald, 2003; Galley et al., 1997; Doise et al., 2008). This is especially true in illnesses, such as septic shock, that exhibit a systemic inflammatory response syndrome (Alonso de Vega et al., 2002 Berger, 2005a; Berger, 2006; Heyland et al., 2013).

The extremely low vitamin C levels in the patients of the current study have already been discussed; and this could have influenced the antioxidant capacity of the patients, as supported by the negative correlation obtained on day 7 between the vitamin C plasma levels and TBARS. It should also be considered that besides being an antioxidant, vitamin C has other associated functions, such as collagen synthesis and endothelial function, which may be at high demand in the early stages of septic shock; and, as such, the levels might not only be explained by the presence or absence of oxidative stress at this stage.

Furthermore, there are other antioxidants (which were not measured in our study) known to be associated with a reduction of oxidative stress – including vitamin E and beta-carotene, glutathione, albumin, and superoxide dismutase. Changes in the levels of these molecules could possibly mask the association between vitamin C and oxidative stress – especially when vitamin C is only present in low concentrations (Berger, 2005; Lovat & Preiser, 2003).

Hyperglycaemia, which is also common and secondary to stress in critical illness, is another contributing factor to oxidative stress (Ceriello et al., 2008; Dungan, Braithwaite & Preiser, 2009). High plasma glucose promotes oxidative stress through the induction of an inflammatory response (Ceriello et al., 2008; Gordin et al., 2008). Patients in this present study had higher plasma glucose levels than the ICU target range at baseline; and this could in part explain the increased oxidative stress.

Considering that increased oxidative stress, if not controlled, causes damage to DNA, RNA, proteins, carbohydrates and unsaturated fatty acids of the cell membranes (Berger, 2005b; Goodyear-Bruch & Pierce, 2002), the patients in this study were at increased risk of cellular and tissue damage. In fact, the increased plasma TBARS at baseline in this study is evidence of lipid peroxidation, a process which, once initiated, may become self-propagating unless terminated, causing cell and tissue injury (Podloucká et al., 2013).

Interestingly, the TBARS levels at baseline were positively associated with the baseline SOFA score, indicating increased organ dysfunction and/or failure. Thus, oxidative stress marked increased organ dysfunction in this study. This result

confirms other previous similar findings, where reduced levels of antioxidants and increased levels of MDA, as well as conjugated trienes were useful as markers of oxidative stress (Biesalski & McGregor, 2007; Heyland et al., 2013; Nepomniashchikh et al., 2009).

Linked to this finding, is the finding that TBARS positively correlates with sVCAM-1, a marker of endothelial damage, which was also high at the baseline. Previous literature has shown that oxidative stress causes damage to the glycocalyx of the endothelial cell (Marechal et al., 2008). Endothelial dysfunction in sepsis has been demonstrated to cause microvascular dysfunction, the motor for sepsis-induced organ dysfunction and failure (Gomez et al., 2014; Piagnerelli, Ince & Dubin, 2012).

Logically, it could be hypothesized that the increased oxidative stress in the patients of the current study influenced the increased incidence of organ dysfunction. On the other hand, the association between oxidative stress and the SOFA score could be a mere reflection of the association between severe SIRS that is associated with septic shock – with resulting organ failure and oxidative stress (REN, 2008). The results from a study by Huet et al. (2007) demonstrated that plasma from septic shock patients induced ROS formation by naive human umbilical vein endothelial cells (HUVEC); and the extent of ROS formation correlated with mortality and with the SOFA score.

The current study points in the direction of a similar conclusion. The baseline oxidative stress marker was significantly higher in the non-survivors than in the survivors in this current study. This demonstrates that even though oxidative damage was evident in the whole group, non-survivors had significantly higher oxidative stress than survivors. However, it was also shown in this study that the non-survivors had increased organ dysfunction, when compared with that of the survivors. These results, therefore, demonstrate that increased oxidative stress is a marker of increased risk of death in this study; and this finding supports the previous similar findings of other researchers (Costa et al., 2013; Huet et al., 2007).

The results of this study also show that oxidative stress persisted at all the time points of the study period (day one TBARS 20.4 nmol/ml (13 – 64); day seven TBARS 13.3 nmol/ml (9 – 18)) with no significant change ($p = 0.52$). Oxidative stress was therefore evident – even a week after the resolution of septic shock. This indicates that the patients in this study were still at risk of the consequences of increased oxidative stress – even after septic shock. It follows, therefore, that early management of oxidative stress in these patients could possibly have saved them from the risk of consequences of oxidative stress, which persists even after septic shock.

Our findings are comparable to those of another study, which reported that patients with septic shock in Chile had persistently increased TBARS – even at day seven of follow up, and were only resolved by three months (Andresen et al., 2008). Careful consideration however should be given when managing oxidative stress in populations at risk, as it is not as simple as merely supplementing with dietary antioxidants.

5.4. Hyperglycaemia and plasma glucose-to-vitamin C ratio

It is understood that septic shock also induces stress hyperglycaemia, which may influence oxidative stress, and also reduce the uptake of vitamin C by endothelial cells – due to the competitive inhibition in sodium transporters (Ceriello et al., 2010; Dungan, Braithwaite & Preiser, 2009). Hyperglycaemia in septic shock could also result from the use of steroids in the management of septic shock, which is a common clinical practice (Fong & Cheung, 2013).

In this study, the median blood glucose for the participants at baseline was 8.84 mmol/l (7.3 – 10.1), which was above the upper limit of the ICU target range (5 – 8 mmol/l). Considering that vitamin C has antioxidant and endothelial functions, hyperglycaemia-induced low vitamin C levels at the endothelial cellular level could influence both the endothelial dysfunction and the oxidative stress. At baseline, the maximum blood glucose in non-survivors had a negative correlation to plasma vitamin C ($r = -0.7$, $p = 0.04$).

A previous study reported that high maximum blood glucose levels during the stay in ICU correlated with increased morbidity and mortality (Christiansen et al., 2004). It follows then, that the control of hyperglycaemia is an essential component in the management of these patients.

In this current study, the median plasma glucose-to-vitamin C ratios at baseline (1756 [998 – 3539]), day one (1178 [727 – 1877]), and at day seven (1043 [593 – 2000]) were above the expected value of 88, calculated from the ratio of the physiological levels of both glucose and vitamin C. The increased ratio could be explained by hyperglycaemia, low plasma vitamin C levels, or by the occurrence of both. In this study, there was hyperglycaemia at baseline, which was controlled at day one and day seven. Vitamin C levels, on the other hand, were persistently low at all of the time points of the study. The hyperglycaemia at baseline and the low levels of vitamin C are the likely explanations for the high plasma glucose-to-vitamin C ratios, persisting up to day seven, following the episode of septic shock.

For the first time, this present study reports increased plasma glucose-to-vitamin C ratios in this patient group. This ratio is important because of its potential influence on endothelial function (since it influences the uptake of vitamin C), and because hyperglycaemia itself is associated with endothelial dysfunction (Chen et al., 2011; Hoffman, Dye & Bauer, 2012; Quagliaro et al., 2005; Su et al., 2013). However, we did not find any correlation between the glucose-to-vitamin C ratio and endothelial dysfunction in this study. This could possibly be due to the fact that in septic shock, profound oxidative stress is what contributes more to endothelial damage, as confirmed by our results (TBARS positively correlated to sVCAM-1), than the influence of this ratio. It is this endothelial dysfunction that is now known to engineer the organ dysfunction associated with septic shock (Donati et al., 2013; Gomez et al., 2014). The next section therefore discusses the findings on endothelial dysfunction in our study.

5.5. Plasma biomarkers of endothelial dysfunction

Evidence from previous studies has shown that oxidative stress causes damage to the endothelium (Sun et al., 2013). We have also discussed in previous sections that vitamin C status and hyperglycaemia may influence endothelial dysfunction (Hoffman, Dye & Bauer, 2012; Zhou et al., 2012). The objective to measure the markers of endothelial dysfunction was therefore important.

5.5.1. Choice of markers of endothelial dysfunction

We used soluble vascular cell adhesion molecule 1 (sVCAM-1) and E –selectin as biomarkers of endothelial dysfunction. Our choice of these biomarkers was based on the fact that both E-selectin and sVCAM-1 are highly expressed during sepsis (Cook-Mills, Marchese & Abdala-Valencia, 2011; Skibsted et al., 2013). However, E-selectin is specific to the endothelium; and it therefore increases in its expression to reflect endothelial activation (Sipkins et al., 2005). It is highly expressed in viable endothelial cells during acute inflammation and increased endothelial proliferation (Kräling et al., 1996; Smadja, Mulliken & Bischoff, 2012).

On the other hand, sVCAM-1 is found in other cells and tissues, in addition to the endothelium (Reinhart et al., 2002). In the endothelium, sVCAM-1 has immunological, mechanical and intercellular transduction functions (Paulus, Jennewein & Zacharowski, 2011). Soluble VCAM-1 mediates leukocyte activation and morphological changes of endothelial cells; and this allows the infiltration of white cells through the endothelium; and it also mediates tissue damage, by release of lysosomal enzymes and the production of reactive oxygen species (Reinhart et al., 2002). Endothelial and tissue damage are hence associated with increased levels of sVCAM-1; and such, it is seen in organ dysfunction and organ failure (Skibsted et al., 2013).

5.5.2. Endothelial dysfunction associated with septic shock

In this study, both sVCAM and E-selectin were high at the baseline. Since it is known that increased inflammatory mediators and endotoxins, which are high in severe sepsis, induce the increased expression of these biomarkers, our results can be understood in this way (Reinhart et al., 2002). The published literature has also shown that hyperglycaemia is associated with the increased expression of sVCAM-1

and E-selectin (Chen et al., 2011; Cook-Mills, Marchese & Abdala-Valencia, 2011); and we have already demonstrated hyperglycaemia in our study group.

At baseline in this study, the sVCAM-1 levels were significantly higher in the non-survivors than in the survivors ($p = 0.01$). This finding could be explained by considering the factors that influence the increased expression of this molecule. Firstly, it is known that sVCAM-1 mediates changes in the morphology of the endothelial cells – to allow for the infiltration of white blood cells into the extravascular space (Hortelano et al., 2010).

In this way, sVCAM-1 promotes a further inflammatory response, which worsens the patient's response to the infection, and predisposes the patient to microcirculatory dysfunction that leads to organ dysfunction and eventual organ failure. The results of this present study have shown that increased oxidative stress is associated with increased sVCAM-1 levels, and sVCAM-1 correlates positively with organ dysfunction. The non-survivors in this study also had increased oxidative stress; and this could be the other explanation for the increase in sVCAM-1 levels in this subgroup of patients. Thus, in this subset of our patients, sVCAM-1 reflected overall worse endothelial dysfunction, illness severity and an increased risk of mortality.

In other studies, increased microbial stimulation has been associated with increased sVCAM-1 expression (Smeding et al., 2012). Considering that the non-survivors had an increased SOFA score and were sicker, it could be hypothesized that they had either had intense infection with an increased microbial load; or they had had a more pronounced inflammatory response, causing increased sVCAM-1 that influenced the severity of their illness – and then caused their death.

In this study, baseline sVCAM-1 was associated with baseline TBARS ($r = 0.45$; $p = 0.02$); and this association has been discussed in a previous section, on the extent and effects of oxidative stress in septic shock. Contrary to the sVCAM-1, there was no significant correlation found between E-selectin and TBARS. The hypothesis was that a positive correlation between these variables would be found. The lack of correlation could be a consequence of the effect of increased oxidative damage to the

endothelial cells, a factor known to negatively affect the E-selectin expression (Harrington et al., 2006).

This explanation would also explain our findings that E-selectin levels were higher in the survivors than in the non-survivors of our study. The non-survivors of septic shock in our study had increased levels of oxidative stress, with increased levels of sVCAM-1, marking endothelial damage. Since endothelial cell damage and necrosis do not induce E-selectin expression (Harrington et al., 2006; Leeuwenberg et al., 1992; Pigott et al., 1992), it is not unexpected to find E-selectin levels higher in the survivors, who were expected to have relatively less endothelial damage than the non-survivors.

The sVCAM-1 levels decreased at day one, and fell within a normal range at day seven, following the cessation of inotropic support for the patients who survived. This demonstrates that there was less cellular damage; and it also indicates recovery in endothelial functions, as septic shock was resolved. This was an expected result. On the other hand, the plasma E-selectin levels remained relatively stable from baseline to day one, but increased significantly at day seven ($p = 0.003$).

This can be explained by the fact that, apart from the stimulation by inflammatory mediators, increased E-selectin expression has been described as a marker of proliferating endothelium; and it is known to be involved in angiogenesis (Kräling et al., 1996; Läubli & Borsig, 2010; Smadja, Mulliken & Bischoff, 2012). The increased E-selectin expression at day seven, following septic shock, may therefore reflect increased endothelial proliferation in repair and regeneration, following the episode of sepsis.

The absence of any correlation between sVCAM-1 and E-selectin at any of the time points of this study could be as a result of a difference in the independent factors that affect the expression of the two biomarkers individually (Cook-Mills, Marchese & Abdala-Valencia, 2011; Cummings et al., 1997; Smadja, Mulliken & Bischoff, 2012). This implies that even though both E-selectin and sVCAM-1 may be increased during inflammation, the expression of these biomarkers could display different kinetics, depending on other dominant processes, such as the endothelial

damage or proliferation that influence their expression (Eikemo, Sellevold & Videm, 2004).

However, yet another study showed significant correlation between sVCAM-1 and E-selectin. The reason for the difference in their result from our own is that their study was in patients with early sepsis; while our study included patients with septic shock, a more severe disease condition associated with organ dysfunction and failure.

5.6. Association between biomarkers and clinical markers

Considering the clinical implications of the vitamin C status and the levels of the biomarkers of oxidative stress and endothelial dysfunction investigated in this study, it was pertinent to investigate the correlation of these biomarkers with clinical markers. We demonstrated in the results of this study that both sVCAM-1 (as a marker of endothelial damage) and TBARS (as a marker of oxidative stress) were positively associated with the SOFA score. The increased sVCAM-1 levels in our study, therefore, marked increased organ failure; while the positive correlation between TBARS and SOFA score supports the hypothesis that septic shock predisposes an individual to overwhelming oxidative stress (Macdonald, Galley & Webster, 2003), both of which induce endothelial damage (De Backer et al., 2013).

We did not find any correlation between vitamin C, at any point of the study, with the SOFA score. We expected a negative relationship. This relationship was perhaps not shown, due to the extreme low plasma levels of this micronutrient in the majority of the patients, which would hardly show any clinical effect. Otherwise, this study finding is difficult to explain.

For the first time, to our knowledge, this study has demonstrated that increased oxidative stress at baseline is associated with an increased requirement for intravenous fluids for resuscitation in a septic shock patient collective. We have shown that oxidative stress is associated with endothelial damage marked by increased sVCAM-1 levels at baseline, and that the endothelial dysfunction is associated with increased organ dysfunction. One of the major organs affected in septic shock is the cardiovascular system, where patients have generalised

hypotension and microcirculatory dysfunction (Dellinger et al., 2013; Hernandez, Bruhn & Ince, 2013).

Such patients require large amounts of intravenous fluids for resuscitation and inotropic support, to bring the blood pressure to physiologically acceptable levels (Dellinger et al., 2013). It could be deduced from this relationship that the level of oxidative stress determined the extent of endothelial damage and cardiovascular dysfunction, hence the amount of intravenous fluid required for resuscitation. However, due to the nature of our study design, we could not establish a cause-and-effect relationship between the oxidative stress and intravenous fluid requirement for resuscitation.

It could be hypothesized that reducing oxidative stress might result in the reduction of intravenous fluid requirements in such a patient group. Tanaka et al. (2000) in a randomized study showed that high doses of vitamin C supplementation in burn patients resulted in reduced oxidative stress levels and lower requirements of intravenous fluids for resuscitation in the patients in the supplementation group. Even though septic shock and burns are different disease conditions, they have common factors, since they both exhibit a profound inflammatory response syndrome, and are associated with increased oxidative stress. One could, therefore, infer that the effect of high doses of vitamin C on the reduction of oxidative stress and the reduction of the requirements of intravenous fluids could also be effective in septic shock patients.

Another unique finding of this study is that increased oxidative stress was associated with more days on inotropic support. This demonstrates that increased oxidative stress in septic shock somehow influences circulatory dysfunction, so that this patient group requires more time on inotropes, presumably due to lingering endothelial dysfunction. Inotrope use is clinically very important, because it reflects illness severity, and therefore the need for organ support and ICU resources.

Getting a patient off inotropes quickly is an indication of an accelerated improvement and response to the other critical aspects of sepsis control, such as source control and antibiotic responsiveness. It follows, therefore, that since the

patients with low oxidative stress in our study had a shorter duration on inotropic support, they were associated with accelerated improvement and response to treatment. Plausibly, management tailored towards addressing increased oxidative stress and its associated pathophysiology could yield a good response in these patients, and hence, a reduction of the duration on inotropes.

5.7. Limitations

The results of this study must be interpreted in the context of its design as a cross-sectional study. In this regard, it can only report associations of variables; but it cannot demonstrate any cause-and-effect relationship. However, the design suits what the study aimed at – to investigate the vitamin C status, oxidative stress, hyperglycaemia, and endothelial function, in critically ill patients with septic shock, without intervening in any way.

Our sample size was 25 patients, and although a relatively small sample, we met the sample size required for the planned statistical analysis.

In this current study, the ferric reducing ascorbate assay for measuring the vitamin C levels in the plasma of the patients was used. High-performance liquid chromatography (HPLC) is currently the gold standard for measuring this micronutrient in plasma. However, the ferric reducing ascorbate assay offers a speedy and reliable alternative for screening plasma ascorbic acid, and it is frequently used in clinical research (Choy, Benzie & Cho, 2003; Chung et al., 2001). Furthermore, this method is relatively cheap and easy to use. Consequently, the use of this method was suitable and cost-effective in the current study.

TBARS was used as a biomarker for assessing oxidative stress. There are other biomarkers of *in vivo* oxidative stress and lipid peroxidation, such as isoprostanes and isofurans (Ware et al., 2011) with higher sensitivity and specificity. Nevertheless, TBARS assay is an assay that has been highly optimised by experienced researchers in our research unit laboratory facility, where this analysis was done; and we therefore defend its use as justified in our setting.

It is recommended in many studies that more than one biomarker assessing the oxidative status be used; and in this current study, this could also pose a limitation.

To investigate endothelial dysfunction, we used circulating (sVCAM-1 and E-selectin, respectively); and we did not have any measures of vascular physiology, such as orthogonal polarization spectral imaging technique or biopsy that could give a picture of the *in situ* processes analysis (Fortin et al., 2010; Mathru & Lang, 2005). Due to the design of the study, *in vivo* assessment procedures were not indicated to assess the endothelium; and the use of circulating biomarkers was considered a suitable proxy for the observational purposes of this baseline study.

5.8. Future directions

Following the results of this observational study, many questions are still to be answered; and further research is definitely indicated. It is a concern that although guidelines exist for the management of septic shock, which are periodically reviewed, the mortality rate of septic shock is still high (Dellinger et al., 2013; Labelle et al., 2012); and this calls for further exploration and the incorporation of other management strategies.

The low vitamin C levels in this patient group definitely require attention. The intervention should also target oxidative stress, which marks increased endothelial damage, the severity of illness, and the risk of death. Antioxidant supplementation would be one of the ways to combat the oxidative stress associated with septic shock. In a meta-analysis of studies that supplemented antioxidant micronutrients in critically ill patients, it has been shown that antioxidant supplementation is associated with a significant reduction in overall mortality among patients with higher risk of death (Manzanares et al., 2012). In a recent, randomised trial of mixed antioxidant micronutrients and glutamine, there was no benefit related to an outcome associated with antioxidant supplementation (Heyland et al., 2013). However, this trial supplemented vitamin C enterally and not parenterally, as some of the studies in the review did (Manzanares et al., 2012). In addition to this, this trial did not study the impact of vitamin C alone. Thus, this area still calls for further exploration and research.

Considering the results of the present study and other previous studies, the role of high-dose vitamin C supplementation warrants exploration, but with caution. Septic shock in our study was associated with the depletion of plasma vitamin C, a phenomenon shared with other settings, where the general population has normal levels of vitamin C. Intravenous vitamin C would be a cost-effective way to replenish plasma vitamin C levels in this patient group, because it is highly bioavailable, compared with enteral delivery (Wilson & Wu, 2012).

It has been demonstrated in other patient groups of critical illness and animal models of septic shock that high-dosage vitamin C restored the vitamin C levels to normal (Gaut et al., 2006; Tanaka et al., 2000; Tyml, Li & Wilson, 2008). These studies further illustrated that high-dose supplementation with vitamin C reduced the oxidative stress, and promoted normal endothelial function. High-dose vitamin C in patients with septic shock could, consequently, be potentially effective in correcting the plasma glucose-to-vitamin C ratio, which was high in our study, by reducing the competitive inhibitive effect on endothelial cell transport by high blood glucose.

In this way, vitamin C could also ameliorate the endothelial dysfunction caused by low vitamin C status. Ultimately, such an adjunctive intervention has the potential to reduce organ dysfunction, as well as the mortality associated with septic shock.

If the potential effects of high-dose vitamin C could be realised, there would then be a potential to reduce the increased requirement for inotropes and intravenous fluids, as discussed in our study. Currently, the use of colloids has been discouraged, due to some poor outcomes associated with their use (Myburgh & McIntyre, 2013; Perel, Roberts & Ker, 2013).

However, this implies that larger volumes of intravenous fluids would be required in the resuscitation of patients with septic shock, since crystalloids are not better volume expanders; and the use of large volumes may cause peripheral and pulmonary oedema (De Backer & Cortés, 2012; Jacob et al., 2012). In addition to this, a reduction in the number of days on inotropic support should reduce the exposure of these patients to the side-effects and complications associated with the prolonged use of vasopressor therapy, such as hypoperfusion (particularly affecting

the extremities, mesentery or kidneys), dysrhythmias, myocardial ischemia, peripheral extravasation with skin necrosis, and hyperglycaemia (Backer et al., 2003; Communal et al., 1998; Singh et al., 2001). This could also perhaps promote quicker recovery, and reduce the cost of such management in this patient group.

Supplementation of vitamin C in patients with septic shock in a South African health setting would be even more justifiable, considering that the general population is at high risk of having low plasma vitamin C levels, when they become critically ill – due to the inadequate dietary intake of vitamin C. Our results showed very low vitamin C status at baseline, which persisted – even after the episode of septic shock. Thus, supplementation ought to be prompt, so that it prevents or reverses early microcirculatory dysfunction and its consequences; and it should be done in parallel with monitoring the oxidative stress status of the patient.

It is with such a rationale that the next phase of this research project will embark on assessing the effects of high-dose vitamin C supplementation on the SOFA score, inotrope requirements, vitamin C status, oxidative stress, hyperglycaemia and the biomarkers of endothelial function in critically ill patients with septic shock.

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APPENDICES

APPENDIX A: Materials, Solutions and Equipment

A1. Materials

96 well sterile plate (Greiner Bio-one, Germany)

Cellstar **R** cryovials (Greiner Bio-one, Germany)

Disposable Pasteur pipettes (Laboratory and Scientific Equipment Company, SA)

Disposable sterile 2ml syringe (Becton Dickinson, UK)

Disposable sterile 5ml syringe (Becton Dickinson, UK)

DNase-, RNase- free 0.6ml polypropylene microfuge tubes (Axygen scientific, USA)

DNase-, RNase-free sterile polypropylene 1.5ml microcentrifuge tubes (Axygen scientific, USA)

EDTA (lavender top) 5ml Vacutainer blood collection tubes (Becton Dickinson, UK)

Heparinised (green top) 5ml Vacutainer blood collection tubes (Becton Dickinson, UK)

A2. Solutions

A2.1 TBARS Assay reagents

Butylated hydroxytoluene (BHT) (Sigma-Aldrich Corporation St. Louis, MO, United States of America)

Ortho-phosphoric acid (Sigma-Aldrich Corporation St. Louis, MO, United States of America)

2-Thiobarbituric acid (Sigma-Aldrich Corporation St. Louis, MO, United States of America)

Ethanol (Merck chemicals (Pty) Limited, Gauteng, Republic of South Africa)

Butan-1-ol (Merck chemicals (Pty) Limited, Gauteng, Republic of South Africa)

Sodium Hydroxide (Merck chemicals (Pty) Limited, Gauteng, Republic of South Africa)

Sodium Chloride (Merck chemicals (Pty) Limited, Gauteng, Republic of South Africa)

Distilled water from (Redox laboratory, human biology, University of Cape Town)

4mM BHT in Ethanol:

Dissolve 0.044g of BHT in 50ml of Ethanol

0.2M Ortho-phosphoric acid:

Dilute 0.137 of Ortho-phosphoric acid in 10ml of distilled water

TBA:

Make 0.1M of NaOH by dissolving 0.8g of NaOH in 200ml of distilled water

Add 0.198g TBA in 12.5ml of the 0.1M NaOH

A2.2 Vitamin C Assay (Ferric Reducing Ascorbate Assay)

Assay done using commercial Ferric Reducing Ascorbate (FRASC) assay kit (Catalog #K671-100) by BioVision Research Products (CA 94043 USA) using prescribed kit protocol.

A2.3 ELISA for soluble vascular cell adhesion molecule (sVCAM-1)

The ELISA for VCAM-1 was performed using a commercial human VCAM-1 ELISA kit (RayBiotech Company, GA 30092, USA) using prescribed kit protocol.

A2.4 Enzyme linked immunosorbent assay (ELISA) for E-selectin

The ELISA for E-selectin was performed using the Human E-selectin ELISA kit (RayBiotech Company, GA USA) using prescribed kit protocol.

A3. Equipment

Digital Dry Bath (Labnet International, Inc, USA)

ELISA plate reader (Biotek Instruments, USA)

Eppendorf centrifuge 5415C (Germany)

Finnpipette digital multi-channel (50-300µl) (Lab Systems, Finland)

Hermle Z100M pulser (Labnet international Inc, Korea)

Refrigerated tabletop centrifuge Hermle Z233 MK-2 (Labnet international Inc, Korea)

Refrigerated Hermle Z233 MK-2 tabletop centrifuge (Labnet international Inc, Korea)

Shaker type 3005 (GFL, Germany)

Tabletop Heraeus labofuge centrifuge (Heraeus Sepatech, Germany)

Tabletop microcentrifuge (Labnet international Inc,Korea)

Vortex Genie 2 (Scientific industries Inc,USA)

APPENDIX B

B1. Patient information and informed consent document

**Patient information and informed consent document for the study entitled:
“Vitamin C status, oxidative stress, hyperglycaemia and endothelial function in
critically ill patients with septic shock: an observational study”**

Investigators:

Lauren Hill*, Kondwani Katundu*, Fahima Adams*, Lester Davids

Division of Human Nutrition, Department of Human Biology, University of Cape
Town

Ivan Joubert, Lance Michell, Malcolm Miller, Jenna Piercy

Division of Critical Care Medicine, University of Cape Town

You are invited to take part in a research study which is being done in the Intensive Care Units (ICU) at Groote Schuur Hospital. The study is about vitamin C levels in patients who have septic shock (severe infection together with very low blood pressure). You are being asked to take part in this study because you have been looked after in the ICU where you were treated for septic shock during your stay in hospital. While you were extremely sick in ICU, some blood samples were taken from you and stored in the freezer for research purposes. Some of the medical information about your health and your recovery in ICU has also been recorded for research. This information includes your blood sugar, blood pressure, the nutrition you received in ICU, and whether you had any organ failure during your illness. All of this information was recorded routinely every day by nursing staff, as part of normal nursing care. The collection of these blood samples and medical information did not put you at any disadvantage in terms of the medical care you needed to recover from being in ICU. Now that you have recovered from your illness, we are asking you to consider giving us permission to use these stored blood samples and the medical information for research.

What is this study about?

When a person is extremely sick in ICU, the body naturally seems to become deficient in certain nutrients, including vitamin C. Vitamin C is known as an antioxidant vitamin. This means that part of its normal functions in the body is to neutralise substances that can be damaging to cells. These cell-damaging substances

(called free radicals) are produced in tiny amounts during normal chemical reactions in cells; and in healthy people, they are quickly removed and kept under control by nutrients, such as vitamin C. However, in severe illness, free radicals can be produced in larger amounts, as part of the body's natural response to being sick. This makes us think that the body has a high need for vitamin C during times of severe illness. In addition, we think that having low vitamin C levels during severe illness may cause the small blood vessels of the body to function more poorly. This might make it more difficult to treat the very low blood pressure that develops in septic shock, and may cause complications of severe illness, such as failure of the organs like the kidneys and the lungs.

What are the tests to be done for research?

In this study, we are interested in measuring the levels of vitamin C in the blood of patients who have had septic shock – to confirm that the body does become deficient in this vitamin. At the same time, we would like to measure the amounts of free radicals that are present, as well as the blood markers that tell us how well the small blood vessels function. While you were in ICU about 3 teaspoons of blood were collected from you every day. during the time your blood pressure was very low (shock). We now ask your permission to analyse this blood. In addition, we would also like to take blood samples from you (about 3 teaspoons each time) once a week until you are discharged from hospital for the same blood tests. We are able to extract the DNA (material in cells containing your genes) from the blood samples. We would also like to keep this DNA for future research on how your genes may allow free radicals to form in larger amounts during illness. We will not use your DNA for *any* other purpose. If you would prefer it, we will not save your DNA for future study. If you request that we do not save your DNA you can still take part in the rest of the study, if you so wish.

What are the risks and benefits to me if I take part in the research?

This study is only about measuring the vitamin C, free radicals and blood vessel function from blood samples. When blood is taken from you, there might be some discomfort, and you may be left with a bruise over the vein. Apart from this, taking part in this study cannot negatively affect your health or put you at risk.

There is also no benefit to you in taking part in the study. The information we learn about vitamin C from testing your blood, might in the future help other patients with severe illness and septic shock in ICU by helping us decide whether vitamin C should be supplemented during illness.

Do I have to pay for the blood tests?

You do not have to pay for any of the blood tests for research (vitamin C, free radicals and blood vessel tests). You will also not be given any payment for taking part in this study.

Do I have to take part in the research study?

No, it is your own choice to take part in this study. If you agree to participate, the blood samples already stored will be analysed; and new blood samples will be taken once a week until you leave the hospital. We will also follow you while you are in hospital and document your recovery or any medical complications you may develop. If you decide not to take part in this study, your blood samples already in storage will be destroyed – without being analysed. All the medical information that has been recorded from your folder for research will also be destroyed. If you choose not to take part in the study, you will still receive any medical care you need.

Will my information be kept confidential?

Yes, all information and research test results will be kept confidential by the study team. Additionally, all the information and results will be labelled with a code, instead of your name, so that your identity is also protected. Only members of the study team will have access to this information. No information that could identify you will be used in publishing the results of the research.

Are my rights protected?

This study has been approved by the Human Research Ethics Committee of the Faculty of Health Sciences, whose role it is to ensure that patients are not put at unnecessary risk during research studies. If you are concerned about your rights, as a research participant, you can make contact with this committee as follows: Prof. Marc Blockman; Chair: Human Research Ethics Committee, phone: 021 406 6626.

If you have any questions about the study itself you can speak to any member of the research team that comes to see you in hospital. You can also phone Dr Lauren Hill on 021 4066769.

In the very unlikely event that you suffer an injury as a direct result of this study, you are covered by an insurance policy held by the University of Cape Town.

If you **agree** to take part in this study, please sign the section below:

I _____ (name) understand that while I was in ICU I became eligible for a research study, and that blood samples for research purposes are in storage. I now confirm by signing below that I take part in this study of my own free will, and I agree that the blood samples and medical information stored for research may be used. I have had all my concerns and questions answered to my complete satisfaction. I have had enough time to carefully consider my participation, and to document my continued participation with my signature.

I understand that my DNA (material in my cells containing my genes) has also been extracted from my blood samples, and is in storage for possible future research on the balance of free radicals in the body.

I ***agree*** that DNA from my blood may be kept in storage for future research ***only*** on the same topic (about free radicals) ☐

I ***do not want*** my DNA to be stored for future research (if you mark this box you can still take part in the rest of the study if you wish) ☐

Signature of participant _____ Date: _____

Name and signature of researcher performing consent procedure:

_____ Date: _____

Name and signature of witness (if applicable):

_____ Date: _____

If you ***do not agree*** to take part in this study, please sign the section below:

I _____ (name) understand that while I was in ICU I became eligible for a research study, and that blood samples for research purposes are in storage. I do not wish to participate in this study, and confirm by signing below that I want the blood samples and medical information stored for research to be destroyed.

Signature of participant _____ Date: _____

Name and signature of researcher performing consent procedure:

_____ Date: _____

Name and signature of witness (if applicable):

_____ Date: _____

B2. APACHE II score

THE APACHE II SEVERITY OF DISEASE CLASSIFICATION SYSTEM

PHYSIOLOGIC VARIABLE	HIGH ABNORMAL RANGE					LOW ABNORMAL RANGE				
	+4	+3	+2	+1	0	+1	+2	+3	+4	
TEMPERATURE — rectal (°C)	○ ≥ 41°	○ 39°-40.9°		○ 38.5°-38.9°	○ 36°-38.4°	○ 34°-35.9°	○ 32°-33.9°	○ 30°-31.9°	○ ≤ 29.9°	
MEAN ARTERIAL PRESSURE — mm Hg	○ ≥ 160	○ 130-159	○ 110-129		○ 70-109		○ 50-69		○ ≤ 49	
HEART RATE (ventricular response)	○ ≥ 180	○ 140-179	○ 110-139		○ 70-109		○ 55-69	○ 40-54	○ ≤ 39	
RESPIRATORY RATE — (non-ventilated or ventilated)	○ ≥ 50	○ 35-49		○ 25-34	○ 12-24	○ 10-11	○ 6-9		○ ≤ 5	
OXYGENATION: A-aDO ₂ or PaO ₂ (mm Hg)										
a. FIO ₂ ≥ 0.5 record A-aDO ₂	○ ≥ 500	○ 350-499	○ 200-349		○ ≤ 200					
b. FIO ₂ < 0.5 record only PaO ₂					○ PO ₂ > 70	○ PO ₂ 61-70		○ PO ₂ 55-60	○ PO ₂ < 55	
ARTERIAL pH	○ ≥ 7.7	○ 7.6-7.69		○ 7.5-7.59	○ 7.33-7.49		○ 7.25-7.32	○ 7.15-7.24	○ ≤ 7.15	
SERUM SODIUM (mMol/L)	○ ≥ 180	○ 160-179	○ 155-159	○ 150-154	○ 130-149		○ 120-129	○ 111-119	○ ≤ 110	
SERUM POTASSIUM (mMol/L)	○ ≥ 7	○ 6.6-9		○ 5.5-5.9	○ 3.5-5.4	○ 3-3.4	○ 2.5-2.9		○ ≤ 2.5	
SERUM CREATININE (mg/100 ml) (Double point score for acute renal failure)	○ ≥ 3.5	○ 2-3.4	○ 1.5-1.9		○ 0.6-1.4		○ ≤ 0.6			
HEMATOCRIT (%)	○ ≥ 60		○ 50-59.9	○ 46-49.9	○ 30-45.9		○ 20-29.9		○ ≤ 20	
WHITE BLOOD COUNT (total/mm ³) (in 1,000s)	○ ≥ 40		○ 20-39.9	○ 15-19.9	○ 3-14.9		○ 1-2.9		○ ≤ 1	
GLASGOW COMA SCORE (GCS): Score = 15 minus actual GCS										
A Total ACUTE PHYSIOLOGY SCORE (APS): Sum of the 12 individual variable points										
Serum HCO ₃ (venous-mMol/L) [Not preferred, use if no ABGs]	○ ≥ 52	○ 41-51.9		○ 32-40.9	○ 22-31.9		○ 18-21.9	○ 15-17.9	○ ≤ 15	

B AGE POINTS:

Assign points to age as follows:

AGE(yrs)	Points
≤ 44	0
45-54	2
55-64	3
65-74	5
≥ 75	6

C CHRONIC HEALTH POINTS

If the patient has a history of severe organ system insufficiency or is immuno-compromised assign points as follows:

- for nonoperative or emergency postoperative patients — 5 points
- for elective postoperative patients — 2 points

DEFINITIONS

Organ Insufficiency or immuno-compromised state must have been evident prior to this hospital admission and conform to the following criteria:

LIVER: Biopsy proven cirrhosis and documented portal hypertension; episodes of past upper GI bleeding attributed to portal hypertension; or prior episodes of hepatic failure/encephalopathy/coma.

CARDIOVASCULAR: New York Heart Association Class IV.

RESPIRATORY: Chronic restrictive, obstructive, or vascular disease resulting in severe exercise restriction, i.e., unable to climb stairs or perform household duties; or documented chronic hypoxia, hypercapnia, secondary polycythemia, severe pulmonary hypertension (>40mmHg), or respirator dependency.

RENAL: Receiving chronic dialysis.

IMMUNO-COMPROMISED: The patient has received therapy that suppresses resistance to infection, e.g., immuno-suppression, chemotherapy, radiation, long term or recent high dose steroids, or has a disease that is sufficiently advanced to suppress resistance to infection, e.g., leukemia, lymphoma, AIDS.

APACHE II SCORE

Sum of **A** + **B** + **C** :

A APS points _____

B Age points _____

C Chronic Health points _____

Total APACHE II _____

B3. The SOFA score calculating sheet

Organ System	Score				
	0	1	2	3	4
Respiratory: PaO ₂ /FiO ₂	>400	≤400	≤300	≤200	≤100
Renal: Creatinine (μmol/l)	≤110	110-170	171-299	300-440;urine output ≤500 ml/day	440; urine output <200 ml/day
Hepatic: bilirubin (μmol/l)	≤20	20-32	33-101	102-204	>204
Cardiovascular: Hypotension	No hypotension	MAP <70mmHg	Dopamine ≤5 ^a , dobutamine (any dose)	Dopamine >5 ^a epinephrine ≤0.1 ^a or norepinephrine ≤0.1 ^a	Dopamine >15 ^a epinephrine >0.1 ^a or norepinephrine >0.1 ^a
Haematologic: Platelet count	>150	≤150	≤100	≤150	≤20
Neurologic: Glasgow Coma scale score	15	13-14	10-20	6-9	<6

For SOFA score: PO₂ : FiO₂ ratio convert PaO₂ in kPa to mmHg by multiplying by 7.5

Use FiO₂ as decimal e.g. if PaO₂ = 8kPa, Convert to mmHg = 8x 7.5 = 60mmHg if FiO₂ = 0.4

60 / 0.4 = 150

^a Adrenergic agents administered for at least one hour (doses given are in μg/kg per minute). FiO₂, fractional inspired oxygen; MAP, Mean arterial pressure; PaO₂, SOFA, Sequential Organ Failure Assessment

(Doig et al., 2004).